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Studies on Erythropoiesis. Part IV. Reticulocyte Response of Hypophysectomized and Polycythemic Rodents to Erythropoietin. (22911)

L. O. JACOBSON, E. GOLDWASSER, L. F. PLZAK, AND W. FRIED

Argonne Cancer Research Hospital, Departments of Medicine and Biochemistry, University of Chicago, Ill.

Erythropoiesis is reduced to a minimum in hypophysectomized rats within 3 weeks after removal of the pituitary(1,2). During this same interval, there is a rapid overall reduction in metabolic requirements(3), although the red cell mass remains within normal limits(4). For this reason, we have suggested that the animal in this state is comparable to one with a transfusion-induced polycythemia and as a consequence, erythropoiesis decreases. Administration of plasma obtained from normal or hypophysectomized animals made anemic by repeated bleeding or phenylhydrazine administration produces an increase in incorporation of Fe^{59} into red cells of the hypophysectomized recipients. The increased incorporation is 3 or more times greater than that in control hypophysectomized animals that are given normal plasma or saline.

The effect of age at the time of hypophy-

sectomy and the effect of transfusion-induced polycythemia upon erythropoiesis are described in this paper. In addition, the reticulocytic response of hypophysectomized rats and polycythemic mice to "anemic plasma" is also reported.

Materials and methods. Anemic and control plasmas were prepared according to the methods described in Part III(5) of this series. *Hypophysectomized rats of 2 age groups.* In one experiment, 30 Sprague-Dawley male rats, 26 to 28 days of age, were studied. The average body weight was 100 g. Reticulocyte determinations were made on all rats the day before 20 were hypophysectomized and the other 10 sham-operated. Thereafter, reticulocyte values were obtained daily from 10 hypophysectomized and 5 sham-operated rats on alternate days. This was continued for 7 days and then at less frequent intervals. In the second experiment,

conducted similarly, the rats were 2 months old with an average weight of 175 g. *Hypophysectomized recipients of anemic plasma.* Four days after hypophysectomy, Sprague-Dawley rats, each weighing about 200 g, were divided randomly into 3 groups, of 5 animals each. Animals in group 1 received no injections (untreated controls); those in group 2, "anemic" rat plasma; and those in group 3, normal rat plasma. Two-cc doses of plasma were injected daily into the tail vein on 3 consecutive days. Reticulocytes were counted prior to the first plasma injection and daily thereafter for 6 days. *Preparation of polycythemic recipients.* CF No. 1 mice weighing 23 g, were made polycythemic by intraperitoneal injections of 75 to 80% concentration of washed homologous red cells suspended in saline. The suspension was given in 0.5-cc doses on successive days and once again 2 days later. Hematocrit determinations were made thereafter at frequent intervals, and red cell injections were repeated when necessary to maintain hematocrit readings of about 70 to 75%. At about 6 days after the first injection, when no reticulocytes were found in the peripheral blood smear, the mice were used for the anemic plasma injection experiments. *Polycythemic recipients of anemic plasma.* Forty-five polycythemic mice were divided into 3 groups of 15 mice each. Those in group 1 received anemic rat plasma; those in group 2, normal rat plasma; and those in group 3, saline. The plasma or saline was injected intravenously for 4 successive days in doses of 0.5 cc. Beginning on fifth day, injections were given every other day until 11th day. In a separate but otherwise identical experiment, blood volume determinations were done on the 3 groups described above as well as on 5 normal mice that received saline and on 5 normal mice that received neither saline nor plasma. In another experiment, intravenous injections of plasma or saline were begun on the same day that mice were given the first intraperitoneal injection of red cells. Plasma was given for 19 days (5 days/week) and the red cell suspension at intervals sufficient to produce and maintain a polycythemic condition. Three groups of 10

mice each were studied. Those in group 1 received anemic rabbit plasma; those in group 2, normal rabbit plasma; and those in group 3, saline. Reticulocyte counts were made frequently throughout the 19-day period of study. In yet another study, doses of 0.5 cc normal or anemic rabbit plasma were given intraperitoneally at the same time that red cells were being given, by the same route, to each of 10 mice in 2 groups. The 10 mice in the third group received saline. Plasma injections were given for 16 days (5 days/week) and the red cells as necessary to produce and maintain a polycythemia. Hematocrit determinations and reticulocyte counts were made on 5, 8, 12, and 15 days following the first plasma and red cell injections.

All hematologic studies were made on blood drawn from tail vein and were limited, for purposes of this communication only, to hematocrits and reticulocytes. Blood for hematocrit determinations was collected in heparinized capillary tubes and spun in a microhematocrit centrifuge.* Reticulocytes were counted by the direct smear method, using brilliant cresyl blue without counterstain. When no reticulocytes were found in 10×10^6 red cells, the values were considered to be 0.000%. Blood volumes were determined according to Cr⁵¹ red cell labeling technic of Sterling and Gray(6). This was modified somewhat for the mice. The radioactivity of each sample was counted in a well-type scintillation counter using 0.1 cc of donor-tagged blood cells in 0.9 cc of distilled water as a standard. Hematocrit determinations were made at the time that blood for blood volume determinations was collected to permit calculation of the red cell mass as well as that of plasma volume.

For histologic studies, polycythemic mice were divided into 4 groups. Group 1 (control), consisted of polycythemic mice that had received neither plasma nor saline. Group 2 consisted of polycythemic mice that had received anemic rabbit plasma. Animals in group 3 had received normal rabbit plasma, and those in group 4, saline. Five mice in

* International Hematocrit Centrifuge, A. S. Aloe Co., St. Louis, Mo.

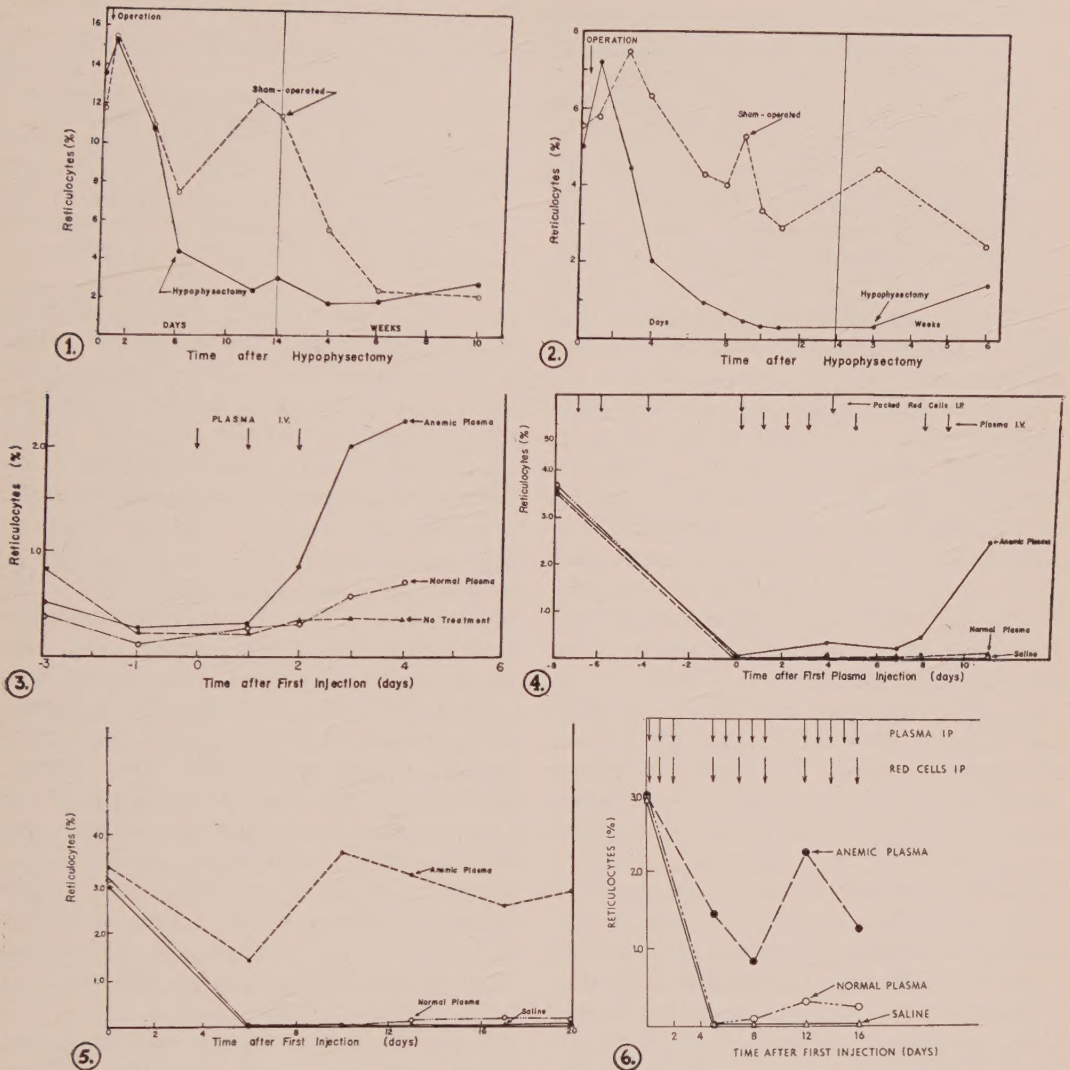


FIG. 1. Effect of hypophysectomy on reticulocyte values of 26- to 28-day-old rats (avg wt, 100 g).

FIG. 2. Effect of hypophysectomy on reticulocyte values of 52-day-old rats (avg wt, 175 g).

FIG. 3. Effect of 2-cc intrav. inj. of anemic plasma compared with that of normal rat plasma on reticulocyte values of hypophysectomized rats (avg wt, 200 g). Injections were started on the 7th day after hypophysectomy.

FIG. 4. Effect of 7 0.5-cc intrav. injections of anemic rat plasma compared with that of normal rat plasma on reticulocyte values of mice that had a transfusion-induced polycythemia.

FIG. 5. Effect of repeated 0.5-cc injections of anemic and normal rabbit plasma on reticulocyte values of mice that were given first intrav. inj. of plasma on same day that transfusions for polycythemia were initiated.

FIG. 6. Effect of repeated 0.5-cc intrav. injections of anemic and normal rabbit plasma on reticulocyte values of mice that received intraper. injections of plasma beginning on same day that transfusions for polycythemia were initiated.

each group were sacrificed 8 days after the first of 6 daily intravenous injections of 0.5 cc plasma or saline. The hematocrit was maintained at about 70% throughout this experi-

mental period. The mice were killed by cervical fracture, and samples of liver, spleen, and bone marrow (femur) were taken for examination. The tissues were fixed in Zen-

TABLE I. Effect of Anemic Plasma on Blood Volume of Normal and Polycythemic CF No. 1 Mice.

Treatment	Wt (g)	Hemato- crit (%)	Total blood vol		Red cell mass (cc)	Plasma vol (cc)
			(cc)	% body wt		
Normal	23	54	1.46	6.3	0.79	0.67
Normal saline	24.6	55.5	1.59	6.4	0.88	0.71
Polycythemic saline	23.9	76.4	2.43	10.1	1.85	0.57
Polycythemic normal plasma	24.4	77	2.58	10.5	1.98	0.59
Polycythemic anemic plasma	24.8	74	2.23	9.0	1.65	0.58

ker-Formol, embedded in nitrocellulose, cut at 6 μ , and stained with hematoxylin-eosin-azure.

Results. Effect of age at time of hypophysectomy. The reticulocyte values of 26- to 28-day-old rats were about 13% on the day before hypophysectomy. During the first 5 days thereafter, the reticulocyte values of hypophysectomized and sham-operated rats were comparable. By 6 days, however, the reticulocyte values of the hypophysectomized rats were appreciably lower than those of sham-operated animals and, as indicated in Fig. 1, they continued to fall until the minimal value of 1.7% was reached by the 4th post-operative week. The number of reticulocytes in sham-operated rats fell more slowly, but by 6 weeks, the values of both groups were comparable and stabilized at 2.5%. Reticulocyte values of 2-month-old rats were about 5.5% before hypophysectomy. The reticulocytes dropped from the pre-operation value to 2% at 4 days afterwards in the hypophysectomized rats and continued to decline, reaching a minimum of 0.3% by 10 days. They remained at this level for 3 weeks, then gradually rose to 1.7% by 6 weeks. The per cent of reticulocytes in sham-operated animals very gradually declined to 2.5 by 11 days after surgery and remained essentially unchanged thereafter (Fig. 2).

Effect of anemic rat plasma on hypophysectomized rats. As indicated in Fig. 3, the reticulocyte values of the hypophysectomized rats injected with anemic plasma rose to 2.3% by the fourth day after the first injection. Values for normal plasma controls reached a maximum of 0.7% whereas those for untreated controls remained at the baseline (Fig. 3).

Effect of anemic plasma on polycythemic

mice. By 1 day following 4 daily injections of anemic rat plasma, the reticulocyte values of polycythemic mice were 0.4% as compared with 0.000% for mice that received similar injections of normal plasma or saline. By 11 days following repeated injections, the reticulocyte values of the group receiving anemic plasma were 2.3%, whereas the reticulocyte values of mice receiving normal plasma or saline were less than 0.001% (Fig. 4). Simultaneous Fe^{59} red cell incorporation studies and reticulocyte counts were conducted in this experiment. On the day that the reticulocytes of the polycythemic anemic plasma recipients were 0.4% and the normal plasma and saline recipients were 0.000%, the Fe^{59} red cell incorporation data failed to show any significant difference between the groups. When reticulocytes reached 2.6% however, the Fe^{59} red cell incorporation studies showed a value more than twice that of the saline or normal plasma controls but failed to show any significant difference between the latter groups.

A definite increase in red cell mass was found in the polycythemic mice that received anemic plasma, normal plasma, or saline (Table I). The plasma volume was decreased moderately in these polycythemic animals as compared with the untreated normal controls. As was expected, no significant difference in red cell mass was noted between the 3 polycythemic groups.

It is worthy of note that a total of only 4 injections of anemic plasma or normal plasma was given in this experiment and that less than 0.001% reticulocytes were observed in normal plasma- and saline-injected groups. In the other experiments described below, many more injections of anemic and normal plasma were given and a slight increase in

reticulocytes above the baseline was invariably observed in the normal plasma-injected animals.

In another experiment, the reticulocyte values of the recipients were 1.3% 6 days after first intravenous injection of anemic rabbit plasma and the first intraperitoneal injection of red cells, whereas these values were 0.000% in mice that received normal plasma or saline. By 10 days, when the reticulocyte values of mice that were given normal plasma or saline were still 0.000% the reticulocytes of the mice that received anemic plasma reached a peak of 3.7%. No further increase in number of reticulocytes occurred in mice receiving anemic plasma, even though the injections were continued for 20 days. A slight increase in reticulocyte values, (about 0.1%) however, occurred in mice receiving normal plasma by 20 days as compared with 0.000% in mice receiving saline (Fig. 5). Injections of anemic plasma given intraperitoneally at the same time that red cell suspensions were injected via the same route produced responses similar to those described above (Fig. 6). By 5 days after the first injections of plasma and red cells, the reticulocyte values of mice that received anemic plasma were 1.4% as compared with 0.000% for mice that had been given normal plasma or saline. By 12 days, the reticulocyte values of anemic plasma-treated animals had risen to 2.2% while those of animals that had been given normal plasma reached 0.25%. The saline recipients remained reticulocyte-free through the 16-day period of observation.

Histologic findings. Histologic differences between polycythemic mice that received anemic plasma and those that received saline or no injection were obvious at autopsy. The spleens of animals that had received anemic plasma were at least twice the size of those from saline-treated or control animals. The spleens from the polycythemic mice that had been given normal plasma were larger than those from the saline or "no injection" controls but smaller than those from the anemic plasma group.

Histologically, the 4 groups were clearly

distinguishable as follows: 1) Anemic plasma-treated polycythemic recipients. Islands of ectopic erythropoiesis in the portal triads of liver were easily distinguishable. Evidence of erythropoiesis was scattered throughout the bone marrow. The red pulp of spleen was packed solidly with erythropoietic tissue (Fig. 7, D). The white pulp was sparsely populated with lymphocytes. 2) Normal plasma-treated polycythemic recipients. Definite islands of erythropoietic activity were found in the portal triads of liver but were less conspicuous than observed in liver of recipients of anemic plasma. The red pulp of spleen had definite areas of erythropoietic proliferation as shown in Fig. 7, B. The amount of erythropoietic tissue in the red pulp was clearly less than that observed in recipients of anemic plasma. 3) Saline-control polycythemic recipients. No indications of ectopic erythropoiesis in liver, no significant evidence of erythropoiesis in bone marrow was seen. The marrow consisted primarily of megakaryocytes and granulocytes. As indicated in Fig. 7, C, the red pulp of spleen was relatively acellular and there was no definite evidence of erythropoiesis. The white pulp stood out sharply in contrast. 4) "No injection" control polycythemic mice. Histologic findings in liver, bone marrow, and spleen in this group were identical with those for saline-injected control mice. A section of the spleen from such a mouse is shown for purposes of comparison in Fig. 7, A.

Discussion. In Part III(5) of this series we have attempted to explain the mechanism whereby hypophysectomy, transfusion-induced polycythemia, and related experimental conditions alter red cell production. We have also tentatively proposed a theory to explain the mechanism by which erythropoietin is produced and by which erythropoiesis is controlled. This communication is largely designed to show that the data on reticulocyte production and the histologic evidence of erythropoiesis in hemopoietic tissue support the findings in the preceding paper in which the incorporation of Fe^{59} into the red cell was utilized to study the state of erythrocyte production as well as to measure the effects of

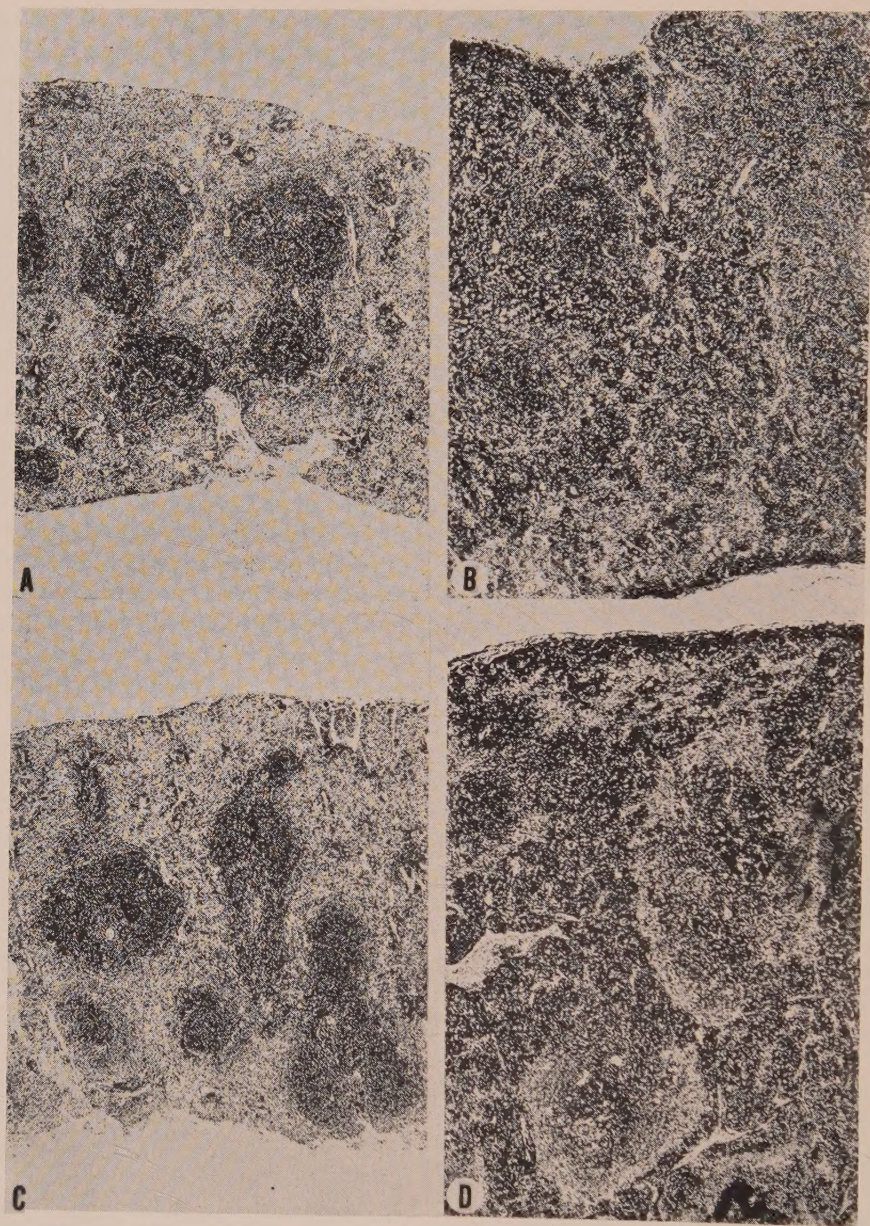


FIG. 7. Histologic effect of 6 0.5-cc injections of anemic rat plasma compared with that of normal rat plasma on splenic erythropoiesis in mice with transfusion-induced polycythemia. Tissues were taken for study 8 days after first inj. of plasma. A. "No injection" polycythemic control spleen. Red pulp is sparsely cellular. B. Spleen of normal plasma-inj. polycythemic mouse. Red pulp is rather heavily populated with erythropoietic tissue. C. Spleen of saline-inj. polycythemic control. Red pulp is sparsely cellular and there are no islands of erythropoiesis. D. Spleen of anemic plasma-inj. polycythemic mouse. There is extreme erythropoietic activity in the red pulp and a moderate reduction in lymphopoietic cells in white pulp.

normal and anemic plasma in hypophysectomized rats and polycythemic mice. It is of considerable importance, however, to note that when one reduces erythropoiesis to an

absolute zero baseline by transfusion-induced polycythemia, as measured by reticulocytes, Fe^{59} uptake values are 5% or less but never reach a zero baseline. We have demonstrated

in polycythemic animals that the response to an anemic plasma preparation may not be significant by Fe^{59} red cell incorporation studies but that reticulocyte studies clearly indicate activity. On the other hand, as described in Part III(5), when a substantial reticulocyte response occurs, Fe^{59} red cell incorporation clearly differentiates the anemic plasma-injected animals from the controls in the polycythemic recipients, and is equally as sensitive as the hypophysectomized assay preparation.

The slight effect of normal plasma on the reticulocytes of the peripheral blood and the striking effect on splenic erythropoiesis probably represents the effect of a small amount of erythropoietin that is present in normal plasma. Our experience would indicate that one could probably use weight, size, and gross appearance of the spleen of polycythemic mice as a simple index of erythropoietic effect of an unknown plasma. The histologic appearance of the spleen permits differentiation between anemic plasma and normal plasma or saline recipients on the basis of the amount of erythropoiesis in the red pulp. In preliminary studies we have found that normal plasma incubated at 37°C for 20 hours and administered thereafter according to the same dosage schedule as that used for normal or anemic plasma in polycythemic mice fails to increase the number of reticulocytes in polycythemic mice.

In his early studies on transfusions, Robertson(7) described the induction of polycythemia in the rabbit and the effect of this procedure on erythropoiesis. He encountered increasing difficulty in maintaining a polycythemia as transfusion followed transfusion. We have found that it is not difficult to produce and maintain a polycythemia in mice if washed homologous cells are used. However, the repeated injection of heterologous plasma makes the maintenance of a polycythemia more difficult.

Summary and conclusions. 1. Following hypophysectomy of rats, reticulocytes in the peripheral blood fall to a minimal level within a period of 3 to 4 weeks. This reduction represents a 5- to 10-fold decrease from the prehypophysectomy control. This reticulocyte decline is more rapid in 8-week-old rats than in younger animals. 2. Administration of anemic plasma to hypophysectomized rats produces a reticulocyte rise that is many fold greater than that produced by normal plasma or saline. 3. Erythropoiesis, as measured by the number of reticulocytes in peripheral blood, falls to zero in 6 days in mice made polycythemic by repeated intraperitoneal injections of homologous red cells. Anemic plasma obtained from rats or rabbits, when injected intravenously or intraperitoneally into polycythemic mice, produces a reticulocytosis many times greater than that observed from normal plasma. Normal saline produces no increase in reticulocytes. 4. In polycythemic mice and hypophysectomized rats, reticulocyte determinations in the peripheral blood and Fe^{59} red cell incorporation studies are of about equal sensitivity in measuring the response to anemic plasma.

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In vitro Sensitivity of Human Leukemic Cells to X-Rays.* (22912)

ROBERT SCHREK, STANLEY L. LEITHOLD AND IRVING A. FRIEDMAN†

Tumor Research Laboratory and Hematology Section, Veterans Administration Hospital, Hines, Ill.

This study explores the feasibility and utility of testing radiosensitivity of blood cells of leukemic patients by time-lapse cinematography and phase microscopy. These methods have been used to determine the effects of x-rays on rabbit(1) and human† lymphocytes and other cells. It was found that lymphocytes treated with small doses of x-rays (50 to 1000r) died with intranuclear vacuolization, lobulation and finally pyknosis of the nucleus. Granulocytes obtained from human blood and from peritoneal exudates of the rabbit, however, were resistant to large doses of x-rays. A few investigators have studied the *in vitro* effects of x-rays on blood cells from leukemic patients. Osgood(2) irradiated fluid tissue cultures of blood cells of one patient with subacute myelocytic leukemia. Irradiation with 300r caused a relative decrease of progranulocytes in 48 and 96 hours presumably due to inhibition of mitosis. Gunz(3) cultivated blood cells from 8 patients with chronic myeloid leukemia and found that 100 and 1000r caused inhibition of mitosis of cells in the cultures but had no effect on resting cells. Schrek(4) tested the effect of 1000r on human leukemic cells by the method of unstained cell counts. Irradiation decreased survival time of blood cells from 4 out of 5 patients with chronic lymphocytic leukemia but had no effect on survival of cells from 7 patients with chronic myelocytic leukemia. According to these 3 studies, irradiation with 1000r or less 1) inhibits mitosis of leukocytes of chronic and subacute myelocytic leukemia, 2) accelerates death of blood cells from most patients with chronic lymphatic leukemia and 3) has no effect on non-dividing leukocytes of myelocytic leukemia.

The methods used in the previous work on

normal cells have been extended to a study of blood cells of 12 patients with chronic lymphocytic leukemia and 6 patients with other types of leukemia.

Methods. To 10 ml of blood from a patient with leukemia, heparin (0.2 ml of 1% solution) was added and the erythrocytes allowed to settle at 37°C for 15 to 60 minutes. The plasma with its suspended leukocytes was then removed. Leukocytes were precipitated by low speed centrifugation, washed twice with Earle's solution and resuspended in a saline medium. The suspension diluted to about 6 leukocytes/ μ ml was mixed with an equal amount of serum, either autologous or of the same blood group. A 0.2 ml drop of the mixture was placed between 2 cover slips separated from each other by a metal ring which formed a chamber 30 mm in diameter and 0.8 mm in depth. The slide preparations were irradiated with an x-ray machine, 100 kv, 5 ma. Doses above 200r were administered at 670 rpm at 10.5 cm from source, and a half layer value of 1.3 mm aluminum. For 50 to 200r, the distance was increased to 31.5 cm to give a rate of 67 rpm. For less than 50r, a $\frac{1}{4}$ mm copper filter was added, the distance was 31.5 cm, and the rate 7.6 rpm. The preparations were examined daily by means of an inverted phase microscope with a fluorite objective, 45x, N.A. 1.0, to observe the number and morphology of surviving cells. In some experiments differential counts were made of viable and dead cells. The number of surviving cells was represented by symbols 5+ to 1+ with 5+ representing survival of all cells and 1+ the survival of less than 5% of the original cells. The differentiation of viable and dead cells was based on direct phase microscopy supplemented by time-lapse cinematography. The viability of leukemic lymphocytes was indicated by their large nuclei, thin nuclear walls, and coarse dark chromatin masses. Some viable cells

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† Chicago Medical School, Chicago, Ill.

† To be published.

TABLE I. Radiosensitivity of Blood Cells from Patients with Chronic Lymphocytic Leukemia.

Patient	Date of test	Survival of lymphocytes, 3 days after irradiation									
		Dosage of x-rays in roentgens—									
		0	4000	1000	500	200	100	50	20	10	5
AM*	8-22	4+	0	0	0	0	1+	2+	2+	2+	3+
WD	-20	3+	0	0	0	0	0	1+	1+	2+	2+
AWC	-13	5+	0	0	0	0	0	0	1+	2+	3+
FJS	-6	4+	1+	2+	2+	2+	2+	2+	2+	2+	3+
AW	7-11	4+	0	0	0	2+	2+	3+	3+		
JSB	6-15	5+	0	1+	1+	3+	3+	4+			
MK	-14	5+	0	0	0	1+	2+	3+			
GS	5-31	4+	0	0	0	1+					
JS	-29	5+	0	0	0	2+					
FS	-16	5+	2+	4+	5+	5+					
MKD	3-23	3+	0	0	0						
AO	-23	4+	0	0	1+						

* Observations made 2 days after irradiation.

5+ to 1+: % of lymphocytes surviving treatment; 5+ represents survival of all or nearly all lymphocytes and 1+ survival of only an occasional cell.

0: No surviving lymphocytes.

had anterior pseudopods and a posterior tail and showed ameboid motion which was accentuated in the cinemicrographic films. Lymphocytes in early stages of degeneration (spontaneous or after 50 to 1000r) were usually lobulated and had one or more large intranuclear vacuoles surrounded by a dark chromatin ring. In latter stages the chromatin ring contracted to a crescent and finally to a round dark pyknotic nucleus. These degenerating and dead cells could be readily differentiated from the morphologically intact, viable lymphocytes. Other types of degeneration and death required recognition and diagnosis by phase microscopy. On addition of fixatives, cells are known to die with only minimal changes in morphology, *i.e.* death by fixation. In this study lymphocytes and other cells irradiated with 4000r were killed in about 3 hours with only minor morphologic changes and this process was termed death by delayed fixation. These changes have been described previously for normal rabbit lymphocytes(5). Here it is only necessary to describe the morphologic features used to diagnose the cells killed by 4000r. In comparison to viable cells, fixed lymphocytes after 4000r had darker chromatin masses and nuclear walls and the nucleoplasm was more profuse and brighter. A few cells had bi- or tri-lobed nuclei. In a few hours the fixed cells underwent autolysis and the autolyzed cells had shrunken dark,

blurred nuclei and profuse transparent cytoplasm. There was some difficulty in recognizing the fixed cells as dead but there was no problem in identifying the autolyzed cell. Further information on the appearance of dead and living cells under phase microscopy is given by Bessis(6).

Results. Chronic lymphocytic leukemia. Blood specimens were obtained from 12 patients with chronic lymphocytic leukemia. In most experiments, nearly all non-irradiated leukemic lymphocytes maintained their morphologic characteristics in slide preparations for 3 to 6 days incubation at 37°C and many cells showed normal ameboid motion during this time. In a few experiments, the leukemic cells survived only 2 or 3 days. The poor survival seemed to be correlated with prior recent x-ray treatment of the patients, but the number of such cases was too small to give conclusive results. In comparison with other studies[†], lymphocytes from leukemic patients with chronic lymphocytic leukemia usually survived as well as lymphocytes from normal blood.

Table I shows that *in vitro* irradiation with 200r killed in 3 days, all or almost all lymphocytes of 11 out of 12 leukemic patients. Even 20r had a considerable cytotoxic effect on cells of 5 patients and 5r caused an appreciable decrease in percentage survival of cells of 3 patients. As can be seen in the Table, 50r killed all the AWC cells in 3 days

and allowed 2+ survival for FJS cells and 4+ survival for cells of JSB. Although further work is indicated, the present data suggest that there is some variability in radiosensitivity of blood cells in these 3 patients. Nearly all FJS cells were killed by irradiation with 20r although a small percentage of cells (2+) resisted even 1000r of x-rays. These findings suggest that the FJS preparations contained lymphocytes of different sensitivities.

We may now turn to a discussion of the unique results obtained with cells of FS. In slide preparations, the FS cells did not differ appreciably from cells of other patients with chronic lymphatic leukemia in morphology, in *in vitro* survival time and in type of spontaneous death. Irradiation of FS cells with 200r or even with 1000r had little or no effect on survival of the cells during 3 days of observation (4+ survival). Irradiated cells continued to die by vacuolization and pyknosis at the same rate as non-irradiated cells. Evidently the degenerative process in these cells was not accelerated by irradiation with 1000r. By time-lapse cinemicrography, the FS cells treated with 4000r were seen to die by the method of delayed fixation in 3 hours.

The findings on the cells of FS were so different that the tests were repeated on 2 additional blood samples with substantially the same results. In addition, the stained smears of peripheral blood and bone marrow were reviewed and the diagnosis of chronic lymphocytic leukemia was confirmed. It may be concluded that FS cells unlike the cells of other patients with chronic lymphocytic leukemia were not affected by irradiation with less than 1000r but gave the same reactions to 4000r as the other leukemic lymphocytes.

The clinical histories of the leukemic patients were reviewed with reference to reaction to x-ray therapy. Five patients received spray x-ray treatments over the anterior and posterior trunk. Table II compares the clinical response of patients to x-ray therapy and the *in vitro* findings on radiosensitivity of the blood cells. As seen previously, the blood cells of 4 patients showed poor survival 3

TABLE II. Comparison of *In Vitro* Radiosensitivity of Blood Cells of 5 Patients with Chronic Lymphocytic Leukemia and Clinical Reaction of Patients to Spray X-ray Treatment.

Patient	Survival of cells, 3 days after 200 r <i>in vitro</i>	X-ray treatment, r	White blood cell count (cells/ μ ml)	
			Before treatment	After treatment
GS	1+	75	210	8.4
FJS	2+	60	137	20
JS	2+	180	182	48
AW	2+	120	350	60
FS	5+	180	580	300

Symbols as in Table I.

days after irradiation *in vitro* with 200r. X-ray treatment of these 4 patients resulted in a rapid decrease of white cell counts, associated with clinical improvement and remission of symptoms. In these 4 patients the leukemic lymphocytes seemed to be radiosensitive both *in vitro* and *in vivo*.

The fifth patient FS had blood cells which have been described as resistant even to 1000r of x-rays. FS was given 6 spray treatments of 30r each or a total of 180r. Following x-ray therapy, the white cell count showed only a minor decrease, the clinical condition deteriorated, and the patient died 2 months after admission to the hospital. The findings suggest a correlation in this patient between the *in vitro* radioresistance of blood cells and failure of patient to respond to x-ray therapy.

The observed radioresistance of leukemic lymphocytes of patient FS suggests that *in vitro* tests may be useful in treating patients with chronic lymphocytic leukemia. Additional tests are being made to determine whether the cells of other patients with chronic lymphocytic leukemia will prove to be radioresistant and whether this information will be useful in outlining treatment and prognosis.

Other leukemias. Blood specimens were obtained from 3 patients with chronic myelogenous leukemia and 3 with acute leukemia. The slide preparations of blood cells showed various types of cells including myelocytes, myeloblasts and lymphoblasts. In the present preparations, none of the immature cells were seen to undergo mitotic division although some cells were observed for long

TABLE III. Radiosensitivity of Blood Cells from Patients with Chronic Myelogenous Leukemia or Acute Leukemia.

Patient	Diagnosis	Date of test	Survival of cells, 2 days after irradiation				
			Dosage of x-rays in roentgens				
			0	4000	1000	500	200
WES	CML	6-6	4+	0	3+	3+	
AM	"	6-13	4+	2+	3+	3+	
AD	"	7-30	3+	1+	3+	3+	
WF	ALL	8-27	3+	0	2+		3+
EB	AML	9-13	5+	2+	4+		4+
ED	"	9-24	5+	2+	4+		4+

CML: Chronic myelocytic leukemia. AML: Acute myelocytic leukemia. ALL: Acute lymphoblastic leukemia.

Other symbols as in Table I.

periods of time by time-lapse cinemicrography. The cells of 3 patients with chronic myelocytic leukemia survived only 3 to 5 days while the cells of 2 patients with acute myelocytic leukemia showed good survival for 7 days.

The effects of x-rays on survival of leukemic cells of this group are shown in Table III which presents observations made 2 days after irradiation. According to previous experiments, most cells of chronic lymphocytic leukemia were killed by 100r of x-rays in 2 days. In contrast, 1000r produced a slight or no decrease in the number of viable cells of patients with chronic myelocytic leukemia or acute leukemia. Irradiation with 4000r killed all or most blood cells of the 6 patients. It may be concluded that the immature non-dividing cells in these preparations, namely myelocytes, myeloblasts and lymphoblasts, were killed by large doses of x-rays but not by small doses. These findings on the effect of x-rays on survival of cells should not be confused with effects of irradiation on other properties of the cells such as mitotic activity.

Summary. 1. This study determined the *in vitro* sensitivity to x-rays of the blood cells of 12 patients with chronic lymphocytic leu-

kemia. The methods used were time-lapse cinemicrography and direct observations with phase microscopy. Non-irradiated leukemic lymphocytes survived 4 to 7 days under the *in vitro* conditions. Dosages of 5 to 1000r reduced survival time of blood cells of 11 out of 12 patients with chronic lymphocytic leukemia. 2. Blood cells of one patient with chronic lymphocytic leukemia were resistant to dosages of 1000r but were killed by irradiation with 4000r. This patient was given clinically 180r spray x-ray treatment but the white cell count showed only a slight decline and the patient failed to respond to the therapy. 3. Irradiation with 1000r produced little or no effect on survival of blood cells from 3 patients with chronic myelogenous leukemia and 3 with acute leukemia.

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Effects in Patients of Intravenous Infusions of Purified Streptokinase Preparations.* (22913)

A. J. JOHNSON, A. P. FLETCHER, W. R. McCARTY, AND W. S. TILLET

Department of Medicine and Surgery, New York University College of Medicine, and Third (NYU) Medical and Surgical Divisions, Bellevue Hospital, New York City.

Tillett, Johnson, and McCarty(1) have described the effects produced by the intravenous infusion of streptokinase (SK) in man. They demonstrated that activation of the blood protease system could be produced by this means, and suggested that this phenomenon might be applied to the therapy of human thrombo-embolic disease. The available streptokinase preparations (Varidase®† assaying at 95 SK units/gamma N) not infrequently evoked reactions of the non-specific protein type consisting of pyrexia, hypotension, and malaise. However, it was demonstrated that premedication of the patients with antipyretic and antihistaminic compounds suppressed the reactions sufficiently to permit infusions of SK in amounts required to create intravascular fibrinolysis. Even though all commercial preparations of Varidase®, for therapeutic use, are devoid of pyrogenicity in rabbits, this type of animal testing has not been a uniformly satisfactory guide to the effect of the preparations when injected intravenously into patients. Previous preparations of streptokinase (contained in Varidase®) were known to be impure, and a study of purification methods has been an integral part of work designed to facilitate further evaluation of induced systemic fibrinolysis in man. It was important to determine, therefore, whether the reactions produced in man by Varidase® were due to impurities in the preparation or to the effects of streptokinase itself.

In the preceding article several methods of purification have been described(2). The purpose of the present investigation has been to test these more highly purified streptokinase preparations for their clinical effect in

man and to indicate their potential advantage for practical therapy. Furthermore, it was desired to induce systemic fibrinolysis in man, without the use of the reaction suppressing drugs previously employed in order to observe the action of the SK activated protease without other conditioning factors.

Materials. Streptokinase purified by four different procedures was available for test (2). *Method 1 material* (assaying at 340-450 units/gamma N) was prepared by chemical fractionation followed by starch zone electrophoresis. *Method 2 material* (assaying at 390-430 units/gamma N) was prepared by a more complex fractionation procedure. *Method 3 material* (assaying at 410 units/gamma N) was method 2 material subjected to one further fractionation step. *Method 4 material* (assaying at 600 units/gamma N) was method 2 material subjected to starch zone electrophoresis.

Methods. Patients suffering from either a local inflammatory lesion, in some cases secondary to arterial or venous insufficiency, or from thrombophlebitis were selected for treatment. The clinical results of the therapy appeared in many instances to be favorable but will be described in a separate communication. The test material was mixed with 500 ml of 1% human albumin-physiological saline solution, and infused at a constant rate over a period of 4 hours. The patient's blood pressure, temperature, pulse and respiratory rates were recorded at hourly intervals, and electrocardiograms were taken, before, during and after treatment on patient's receiving 30,000 or more units of streptokinase. No drugs were administered during the infusion or observation period. The presence or absence of fibrinolysis in the peripheral blood was determined by previously described methods(1).

Results. The present studies made with the different purified preparations have been

* This study was supported by grants from N.I.H., and Lederle Laboratory Division, American Cyanamid Co.

† Streptokinase-Streptodornase, Varidase®, Lederle Laboratories, Pearl River, N. Y.

TABLE I. Effect of Varying Doses of Different Lots of Purified Streptokinase in Man (128 Infusions in 61 Patients). Figure in front of slash in each column on the right shows No. of infusions at each dose level, and figure after slash shows No. of infusions that caused a clinical reaction. The figure in parentheses indicates No. of reactions that were of moderate severity (see text for criteria).

Method of preparation			No. of patients	Dose in SK units		
Method	Lots	Units/ γ N		10,000-30,000	31,000-70,000	71,000-130,000
1	4	340-450	21	16/ 4	4/6	2/2 (1)
2	2	390-430	9	17/10 (2)	—	—
3	1	410	16	30/ 8	5/4 (1)	5/2 (2)
4	2	600	15	28/ 6	3/0	8/3 (3)

compared with the effects produced by intravenously injected Varidase®. In the previous report(1) on intravenous Varidase®, the patients received relatively large doses (80,000 to 150,000 units) and the febrile and hypotensive effects of the infused material were suppressed by the appropriate administration of amidopyrine and chlor-trimeton.

In addition, extensive previous experience with intravenous Varidase® (not reported in detail) without the use of suppression drugs and in wide ranges of dosage has shown that 2 out of 3 patients experienced a 2-3°F rise in body temperature, and 1 out of 4 patients experienced a fall of blood pressure following the infusion of 5,000-10,000 units of streptokinase. A dose of 10,000-20,000 units produced some pyrexia and hypotension in nearly all patients, together with malaise. A rigor was sometimes produced, while malaise, nausea and vomiting were occasionally noted in the absence of pyrexia. All symptoms subsided within 12 to 24 hours.

I. *Comparison of effects of preparations made by each of 4 methods.* Three grades of reaction to the test infusion were recognized. The patients were assumed to have had no reaction, if no symptoms or signs were detected following the infusion. A *mild reaction* was diagnosed if the temperature was raised 1°F, if the systolic blood pressure fell 20 mm of mercury or the diastolic blood pressure fell 10 mm mercury, or there were mild untoward symptoms. A greater rise of body temperature (more than 1°F), a greater fall of blood pressure, or untoward symptoms other than mild were regarded as indicative of a *moderate reaction*. Patients who experienced moderate reactions

were usually treated with antipyretics, or sometimes with both antipyretics and antihistaminics after termination of the infusion. Such therapy produced rapid symptomatic relief with a prompt fall of body temperature to normal limits. Patients tended to react less to a second injection of streptokinase than to a first injection. This source of error does not invalidate the comparison between the various batches of material tested as the phenomenon was inconstant and approximately equal numbers of first and second infusions were made in each group.

The results with 4 different lots of SK, purified by different methods, have been tabulated at 3 dose ranges: low, 10,000 to 30,000 units; moderate, 31,000 to 70,000 units; high, 71,000 to 130,000 units. These ranges were selected so that the majority of patients in each group received approximately the mean dosage in each range. The infusion data for the various lots are summarized in Table I.

Table I shows that the toxicity of material prepared by method 2 was greater than that prepared by the other 3 methods. The data are insufficient to decide between the merits of the other 3 methods though they suggest that method 4 was the best. The incidence and degree of pyrexia, hypotension and untoward symptoms experienced by patients who received 100,000 units of material prepared by methods 1, 3 and 4, was closely similar to that experienced by patients who received 5,000 units of Varidase®. For this reason, it was concluded that the purification of streptokinase, in the manner described, had reduced its toxicity to man by approximately 95%.

TABLE II. Clinical Effect of Systemic Fibrinolysis. Following infusion of comparable amounts of streptokinase, one group of patients (lysis group) developed an active lytic system in the blood, while the other did not (control group). Clinical response of these groups is shown in table (for criteria of reactions see text).

Clinical effect	Control group (no lysis)	Lysis group
	No. of infusions	
No reaction	12 (35%) \ 25 (73%)	4 (27%) \ 13 (87%)
Mild "	13 (38%) /	9 (60%) /
Moderate reaction	9 (27%)	2 (13%)
Total No. of infusions	34	15
No. of patients	26	12

Caution was observed in the clinical trials of each of the preparations and as a consequence, the majority of patients received insufficient quantities of streptokinase to activate their blood protease systems. However, high levels of fibrinolytic enzyme were detected in the blood of 15 patients following the streptokinase infusion. Blood, withdrawn from the patients at the end of the infusion and for some hours afterwards, showed spontaneous clot lysis in 45-240 minutes(1). The symptoms and signs shown by these patients who received streptokinase and exhibited fibrinolytic activity in the blood stream, were compared to a similar group, who received the same dose of streptokinase but who failed to show clot lysis (Table II). Though the number of observations is small, it appears that the two groups experienced a similar incidence and degree of clinical reaction. The fact that 13 out of 15, or 87%, of the infusions in the patients with lysis were followed by no more than a 1°F rise in temperature, or a small fall of blood pressure suggests that high levels of circulating fibrinolytic activity are tolerated in man without significant symptomatology.

Electrocardiographic studies. Since intravenous infusions of SK will be employed as specific treatment in selected patients with cardiovascular disease, a number of patients who received infusions of streptokinase, prepared by these and other methods, were followed with extensive electrocardiograms. Kellner and Robertson(3) recently demonstrated that injection of large doses of streptokinase into rabbits caused cardiac damage.

Neither clinical nor electrocardiographic examination of these patients have shown evidence of such an effect in man. 165 electrocardiograms were taken on 60 patients, most of whom were in the older age groups. Standard limb leads, unipolar extremity and precordial leads were recorded by means of a Cambridge string galvanometer. An electrocardiogram taken prior to infusion of streptokinase acted as control for subsequent tracings taken on each patient during, immediately after, or within 12 hours following the infusions.†

No abnormality was evident before or after the infusions in 114 electrocardiograms taken on 47 of these patients free of heart disease.

It seemed particularly significant that there was no change in 31 abnormal electrocardiograms taken before, during and after the SK infusions on 9 patients who had heart disease. This group included 2 patients with severe hypertensive and arteriosclerotic heart disease and 5 with moderately severe arteriosclerotic heart disease complicated by recurrent congestive heart failure and/or cardiac arrhythmias. The electrocardiogram of another patient showed premature ventricular systoles and inverted T waves in leads I, II, AVL, V3 and V5 with no other evidence of cardiac disease. The last patient in this group, with moderately severe heart disease

† The authors gratefully acknowledge their indebtedness to Dr. Bertha Rader of N. Y. University-Bellevue Medical Center for electrocardiographic interpretations.

of unknown origin, had congestive heart failure on three different occasions and was on a maintenance dose of digitalis. The average age of these patients was 67 years.

The electrocardiograms of 4 other patients who had abnormal T-waves in their control tracings showed minor changes in these abnormalities during the period of observation and infusion. One of these patients had no previous evidence of heart disease except an electrocardiographic abnormality, one had hypertensive and arteriosclerotic heart disease and 2 had arteriosclerotic heart disease. The tracings on one of the patients with arteriosclerotic heart disease had deeply inverted T waves in V3 before the infusions which became less inverted during and after the infusions, while those on the patient with hypertensive and arteriosclerotic heart disease had low T waves in nearly all leads which also became more positive during the infusion period. Similarly, the non-cardiac patient had an inverted T in lead AVL in his control tracing which became more positive as the infusions progressed. The last patient was found to have frequent premature ventricular contractions prior to, and following the infusions and a short run of ventricular tachycardia on one of the 9 infusion days. These changes were not associated with any symptoms, the amount of SK administered or with simultaneous laboratory studies including the serum oxaloacetic transaminase.

In short, the few changes that did occur in these electrocardiograms were minor and inconsistent and therefore could not be ascribed to the effect of the infusion.

Discussion. The clinical trial, during which 128 infusions of streptokinase were administered to 61 patients, shows that the toxicity of streptokinase to man can be greatly reduced by purification. It appears that methods 1, 3 and 4 produce material that is only 1/20th as toxic to man as is Varidase®. The toxicity comparison between methods 1 and 2, both producing material of almost identical potency but of differing clinical toxicity, suggests that toxicity may be unrelated to the administration of streptokinase as such, but

rather due to the presence of a contaminating substance. Immunochemical analysis has shown that all the present preparations contained more than one antigenic component, though a positive identification of these antigens has yet to be made.

The observation that high levels of circulating fibrinolytic enzyme produce no symptoms in man is of theoretical interest as well as of practical importance. It has been repeatedly suggested (Reviews 4, 5) that activation of the blood protease system, leading to the liberation of biological split products (anaphylatoxins) was the cause of, or played an important role in the production of the hypersensitivity reaction. The demonstration that histamine release and blood protease activation occurred during this state after union of circulating antigen with sensitized tissue(6,7) that antigen-antibody union may activate serum protease *in vitro*(8), and that in some instances, at least, proteolysis and histamine release were correlated in time and amount(9) have maintained interest in this mechanism. Our findings, that activation of the blood protease system in man is not accompanied by clinical symptomatology, seem to exclude participation of the plasminogen-plasmin system in the pathogenesis of the hypersensitivity reaction.

Summary. 1. The clinical effects of specially purified streptokinase preparations have been determined in man. Streptokinase, 6-7 fold purer than that previously available, has been shown to have a greatly diminished toxicity, when administered by the intravenous route. The best of these preparations have only 1/20th of the toxicity of the previous preparations. 2. Systemic fibrinolysis has been induced in man by infusion of these preparations, unprotected by reaction suppressing drugs. A comparison between those patients who showed peripheral blood fibrinolysis, and those who did not, indicated that this state is asymptomatic in the human. 3. 165 electrocardiograms taken on 60 patients, before, during and after the infusion of streptokinase showed few changes and these were of minor degree. Since they followed no consistent pattern it was not pos-

sible to ascribe the changes to the effect of the infusion.

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Cardiotonic Activity of 9-Alpha Fluorohydrocortisone.* (22914)

RALPH D. TANZ,[†] RICHARD W. WHITEHEAD, AND G. J. WEIR, JR.

Department of Pharmacology, University of Colorado School of Medicine, Denver.

Previous work from this laboratory(1) reported the positive inotropic action of 9-alpha fluorohydrocortisone[‡] (9- α FF), and its effect upon Na²² distribution in the papillary muscle preparation. In the present paper evidence is presented showing the positive inotropic action of 9- α FF in low concentrations, and its negative inotropic action in higher concentrations. These findings are correlated with histological changes in cardiac muscle and intramuscular Na²² and K⁴² movement.

Methods. 1. *The cat papillary muscle preparation* first described by Cattell and Gold(2) was employed with certain modifications(1). Briefly, 2 muscles were isolated from the right ventricle without causing gross injury. A control and a treated muscle were stimulated in two identical chambers at 60 times per minute throughout the experiment. Krebs-Ringer bicarbonate solution enriched with dextrose was used as the bathing fluid maintained at 38°C and a mixture of 95% O₂ and 5% CO₂ bubbled through it. A minute amount of either Na²² or K⁴²§ was added

to the bathing fluid, the amount of K⁴² added being taken into account when preparing the medium. 9- α FF in amounts indicated in Table I was then added to the bathing fluid of one of the muscles. The uptake of isotopic Na or K was expressed in counts/sec/mg and then in terms of the ratio of radioactivity of the samples treated with 9- α FF to that of control samples. 2. *The isolated heart preparation* as modified by Anderson and Craver (3) was employed using whole cat hearts and enriched Krebs-Ringer bicarbonate solution as the perfusing medium. Failure was induced by perfusing the preparation with fresh, unused perfusate(4). Since 9- α FF in a concentration of 0.5 μ g/ml caused a positive inotropic action in the papillary muscle preparation, this concentration was added to the reservoir bottle and perfused through the heart after signs of cardiac failure appeared. 9- α FF 'toxicity' was induced by adding 10 μ g/ml to the perfusate at 0 time, as indicated in Fig. 2. 3. *Histologic sections* were obtained from fresh papillary muscles or those stimulated at 60 times per minute for 6 hours in concentrations of 0, 0.5 or 20 μ g/ml

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[†] U.S.P.H.S. Fellow of N.H.I.

[‡] Acknowledgement is made to Merck & Co., Rahway, N. J., who provided us with 9-alpha fluorohydrocortisone.

§ Specific activity of Na²² = 0.75 μ C/ml (and amounted to 0.48 mEq/l in 143 mEq/l of Na); specific activity to K⁴² = 0.047 μ C/ml (and amounted to 0.4 mEq/l in 5.66 mEq/l of K).

TABLE I. Results Obtained with 9-Alpha Fluoro-hydrocortisone.

Exp.	Drug conc. ($\mu\text{g/ml}$)	Iso- tope	Ratio:* Exp./C†	Δ inotropic action (%)‡
1-11	.5-1.0	Na^{22}	>1	all positive§
12	C†	K^{42}		
	.5		.57	+439
13	C			
	.5		.89	+219
14	C			
	.5		.96	+ 87
15	C			
	.5		.95	+105
16	C	Na^{22}		
	10		.77	- 52
17	C			
	10		.53	- 57
18	C			
	20		.66	- 22
19	C			
	20		.82	- 91
20	C	K^{42}		
	10		1.06	- 89
21	C			
	20		1.44	- 23
22	C			
	20		1.60	- 37
23	C			
	20		1.15	- 68

* In counts/sec./mg.

† C = Control.

‡ Measured from just prior to administration of drug to highest or lowest point reached after administration.

§ See Tanz *et al.*(1).

of 9-*a* FF. Typical sections are compared in Fig. 3.

Results. Fig. 1 shows the effect of adding 9-*a* FF to the perfusing fluid of the papillary muscle preparation. Average values of these experiments, which were measured at 20 minute intervals, are plotted in terms of % height of the initial contraction. The upper dotted line represents results obtained on 7 muscles using a concentration of 0.5-1.0 $\mu\text{g/ml}$ (1). The lower dotted line shows results obtained in a range of 10-20 $\mu\text{g/ml}$ on 7 muscles. These results show a positive or negative inotropic action (depending on dosage), when compared to mean control values represented by the solid line(1).

Results from individual experiments are tabulated in Table I. In the 0.5-1.0 $\mu\text{g/ml}$ range a positive inotropic action is found

along with an increased uptake of Na^{22} and a loss of K^{42} , in comparison with its control from the other chamber. In the 10-20 $\mu\text{g/ml}$, or 'toxic' range, a negative inotropic action is obtained with a loss of Na^{22} and an increased uptake of K^{42} , in comparison with its control.

Addition of 0.5 $\mu\text{g/ml}$ to the perfusate of the failing isolated heart preparation resulted in partial recovery within 40 minutes. Conversely, addition of a 'toxic' dose of 10 $\mu\text{g/ml}$ (Fig. 2) brought about cardiac failure in about 15 minutes.

Histologically, sections of the fresh papillary tissue show good cellular integrity, lightly stained elongated nuclei and typical cross-striations of cardiac tissue. The tissues stimulated continuously for 6 hours in a concentration of 0.5 $\mu\text{g/ml}$, show fairly good cellular integrity, slightly darker elongated nuclei and an impression of cross-striations. Conversely, control muscles and those treated with 20 $\mu\text{g/ml}$, stimulated for the same period of time, are greatly swollen with pyknotic and warped nuclei, a foamy cytoplasm and loss of cross-striations; all signs of

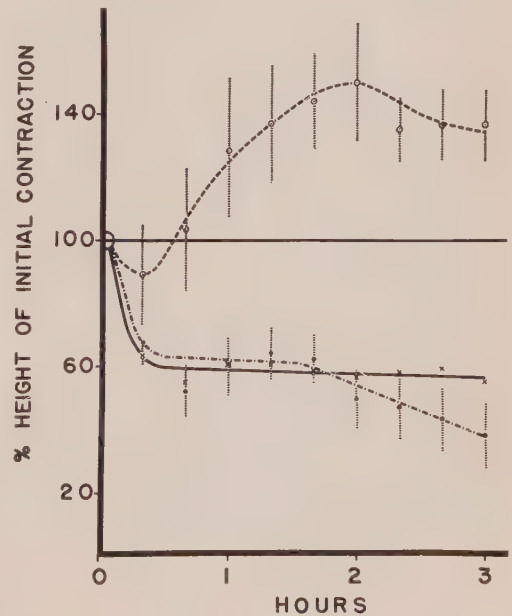


FIG. 1. Dotted curves show inotropic action when varying concentrations of 9-*a* FF are added. Upper curve = 0.5-1.0 $\mu\text{g/ml}$; lower curve = 10-20 $\mu\text{g/ml}$. Vertical lines represent the calculated stand. errors. Solid curve shows mean of 11 control experiments.

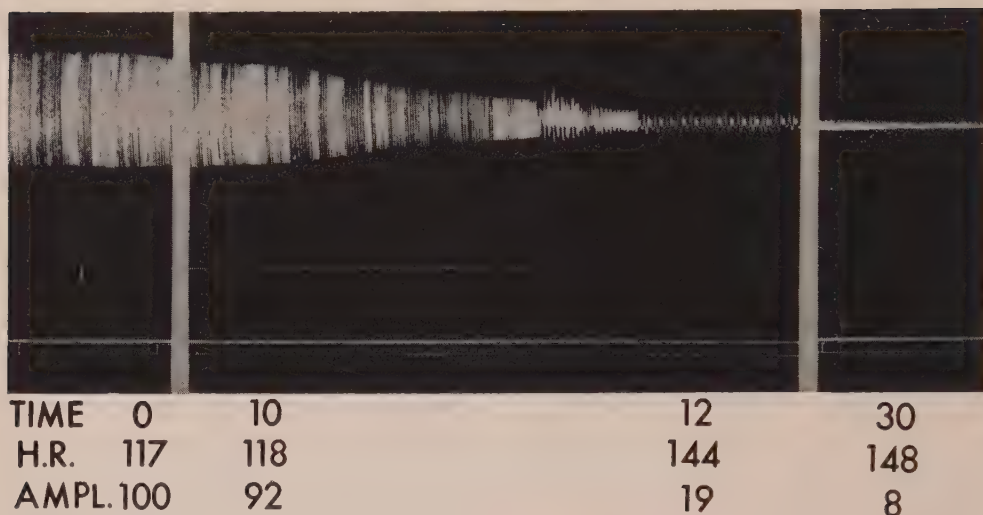
9-*a* FF 10.0 μ g/ml.

FIG. 2. Kymographic recording using Anderson-Craver heart perfusion apparatus showing 9-*a* FF 'toxicity.' 10 μ g/ml of 9-*a* FF added at 0 time. (Time markings in 10 sec. and 1 min.; TIME in min.; AMPL. in %.)

cardiac degeneration.

Discussion. These results are presented as evidence of the cardiac glycoside-like action of 9-*a* FF on isolated heart tissue. The evidence that Gowdey *et al.* (1950) and previous workers(5-11) have presented using a wide variety of steroids, indicated to us that some might possess a direct glycoside-like action on cardiac tissue. The fact that the steroid nucleus is common to both cardiac glycosides and steroids lent further support to this belief.

Using the 8 K^{42} and 4 Na^{22} control experiments from Table I, it was calculated that there was about 9.5 times as much K^{42} as Na^{22} in the control muscles at the end of 3 hours. Although there were too few experiments for a quantitative measurement, it is certainly indicative that a positive K/Na ratio did exist in the controls. The fact that this ratio was not greater than 9.5 is in agreement with Fenn(12,13) and others(14,15) who have stated that muscles take up Na and extrude K during contraction.

When experimental muscles were given a 'therapeutic' dose of 9-*a* FF (0.5-1.0 μ g/ml), and a positive inotropic action resulted, the ratios in Table I show that they took up Na^{22}

and lost K^{42} . Conversely, a 'toxic' dose of 9-*a* FF (10-20 μ g/ml), resulted in positive K^{42} and negative Na^{22} ratios, while showing a negative inotropic response. This is in agreement with Korol (1956), who showed that cardiac depression is associated with loss of Na and an uptake of K and Gertler *et al.*(16) who showed that quinidine toxicity produced intracellular hyperpotassemia, and possibly intracellular hyponatremia.

Hajdu and Szent-Gyorgyi(8) have shown that variations in physiological response of isolated cardiac tissue might be due to the washing out of some substance by the perfusion fluid. The addition of serum helped restore a normal physiological response, and partial identification of the responsible substance(s) in serum indicated a steroid type of compound. Furthermore, Leitch†(4) has observed that the original perfusate must be recycled through the isolated heart preparation to prevent failure. On the basis of our results with the isolated heart perfusion apparatus, we believe that one or more naturally occurring steroids are necessary for normal cardiac function. The fact that such small amounts of 9-*a* FF are needed to elicit a re-

† Personal Communication.

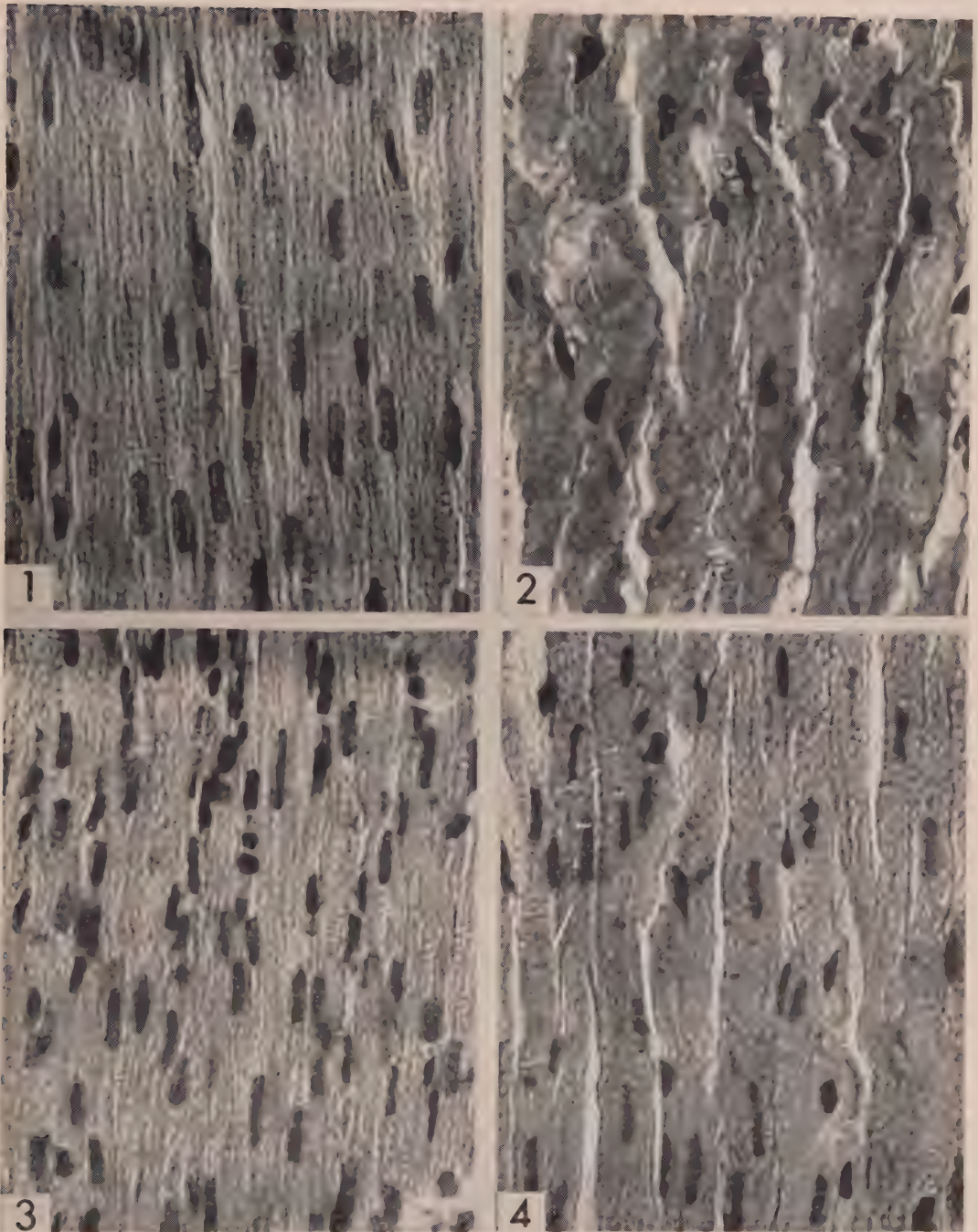


FIG. 3. Photomicrographs of papillary muscles. (1) Fresh tissue. (2) Control tissue stimulated 6 hr. (3) Tissue treated with 0.5 $\mu\text{g}/\text{ml}$ of 9-a FF and stimulated 6 hr. (4) Tissue treated with 20 $\mu\text{g}/\text{ml}$ of 9-a FF and stimulated 6 hr. (H & E $\times 1000$.)

sponse is indicative of a specific action on cellular activity, probably exerting its influence by altering membrane permeability.

Summary. (1) Addition of 9-a FF in a con-

centration of 0.5-1.0 $\mu\text{g}/\text{ml}$ results in a positive inotropic action, an increased uptake of intramuscular Na and loss of K. (2) In a concentration of 10-20 $\mu\text{g}/\text{ml}$, 9-a FF gives

rise to a negative inotropic action, loss of intramuscular Na and an increased uptake of K. (3) Employing the isolated heart preparation, addition of 0.5 $\mu\text{g/ml}$ of 9-a FF to the failing heart results in a partial recovery. Conversely, 10 $\mu\text{g/ml}$ added to the 'normal' heart brings about failure quite rapidly. (4) A histological correlation of the reported results is shown. (5) The mechanism of action and significance are discussed.

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A Cytopathogenic Agent Isolated from Naval Recruits with Mild Respiratory Illnesses.* (22915)

W. PELON,[†] W. J. MOGABAB,[†] I. A. PHILLIPS AND W. E. PIERCE
(Introduced by Laurence W. Roth)

Virology Division, Naval Medical Research Unit No. 4, Great Lakes, Ill. and Division of Infectious Disease, Department of Medicine, Tulane University School of Medicine, New Orleans, La.

A cytopathogenic agent has been isolated in monkey kidney cultures from nasopharyngeal washings obtained from Naval recruits with mild respiratory illnesses(1). In this paper, certain characteristics of the isolate are described which allow preliminary classification and identification. The relation of infection to clinical illness in 2 recruit companies and evidence suggestive of widespread

infection in other populations are also presented.

Methods. Cultures were prepared with 0.25% trypsin (Difco, 1:250) dispersed kidney cells from adult Rhesus monkeys at a concentration of 500,000 cells per ml. These were incubated at 35°C in a stationary position in nutrient medium consisting of 90% mixture 199(2) (Difco Laboratories) or bovine amniotic fluid (Cudahy), 5% inactivated horse serum (56°C for 30 minutes), 5% bovine embryo extract(3), penicillin, 200 units and streptomycin, 200 μg per ml. The same media or mixture 199 alone plus antibiotics was used when the cultures were ready for inoculation. Monkey testis, cornea, human embryo kidney and chick embryo cultures were propagated similarly. HeLa, KB

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[†] Present address: Department of Medicine, Tulane University School of Medicine.

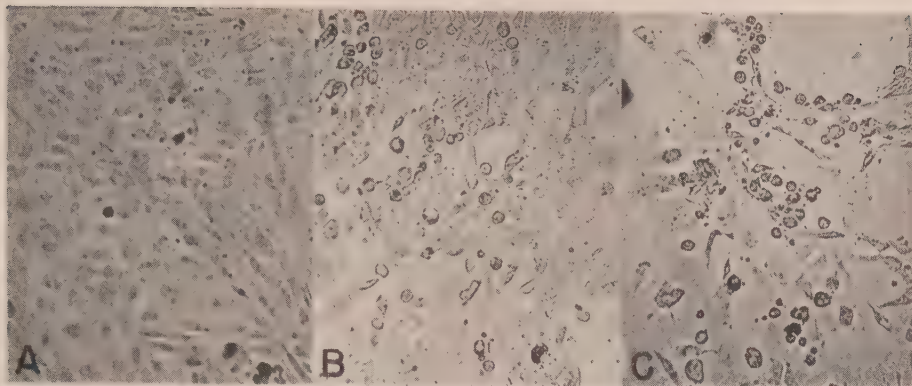


FIG. 1. Cytopathogenic effects of 2060 in monkey kidney cultures. (A) Normal, (B) 5 days after inoculation of 10^8 TCID₅₀, (C) 10 days after infection (55 $\times$).

(4), and human conjunctival cells(5) were cultivated in Scherer's maintenance solution (MS)(6) (Microbiological Associates), containing 40% pooled human serum, washed and maintained for inoculation with Scherer's MS containing 7.5% chicken serum and 25% tryptose phosphate broth(7). Infectivity (TCID₅₀) was determined by inoculating 0.1 ml of 10 fold dilutions of the agent into groups of 4 monkey kidney cultures. Readings were usually made at 10 days on the basis of cytopathogenic effects. Neutralization was done with inactivated sera (56°C for 30 min.) which were held with the agent at 4°C for 30 minutes prior to inoculation of cultures. Readings were made at the end of 10 days and titers determined on the basis of inhibition of cytopathogenic effects. Tissue culture fluids were not changed after inoculation of the agent-serum admixture.

Results. Primary isolation. Cytopathogenic effects were observed upon second monkey kidney culture passage of nasal washing No. 2060 obtained from a recruit with a mild respiratory illness in October 1954. A probable relation to infection by the agent in this individual was demonstrated by increase in neutralization titer from less than 8 in the acute serum to 56 in the convalescent serum. Isolation was repeated in kidney cultures from a different monkey inoculated with another aliquot from the same specimen. In both instances, characteristic cellular changes occurred 8 days after inoculation of the second group of cultures. Serial passage of

the isolate was carried out in monkey kidney cultures.

Cytopathogenic effects. The isolate produced characteristic changes in monkey kidney cultures, manifested first by enlargement and rounding of epithelial cells, fine cytoplasmic granularity and indistinct nuclei (Fig. 1b). Involved cells later became pyknotic and fragmented (Fig. 1c). Cytopathogenic changes were not found prior to 3 days after inoculation of undiluted material. After 12 passages of the agent, complete destruction of epithelial cells occurred only after a period of 10-14 days. Many of the disrupted cells remained attached while others became loosened from the walls of the culture tube. Intracellular or cytoplasmic inclusion bodies were not found in Giemsa stained cells. Cells treated early in the course of infection by means of the indirect fluorescent antibody technic of Weller and Coons(8), revealed an accumulation of fluorescing granules in the cytoplasm adjacent to the nucleus.† Staining of the entire cell occurred as infection progressed.

Classification. The relation of 2060 to known viruses was first investigated by the effects in various tissue cultures and animal hosts. The original nasal washing as well as the 12th monkey kidney passage with a TCID₅₀ of $10^{7.0}$ were used in separate experiments. Several blind passages were also

† Fluorescein isocyanate conjugated antihuman gamma globulin rabbit serum was kindly supplied by Dr. A. H. Coons.

made. Cytopathogenic effects were found in cultures of monkey testis and human embryo kidney, but not in monkey cornea, chick embryo, HeLa, KB or human conjunctival cells. Inoculation of the yolk sac, chorioallantoic membrane, amniotic or allantoic sacs of 6- to 10-day embryonated eggs did not produce visible lesions, deaths or hemagglutinins. Swiss mice less than one day or 3 weeks old were not affected by serial intracerebral, intranasal, or intraperitoneal passages. Effects were not seen in rabbits inoculated into the cornea, peritoneal cavity, or intravenously.

Antisera utilized to further exclude some viral and rickettsial agents included poliomyelitis, types 1, 2, and 3 (simian); adenoviruses, types 1 through 7 (rabbit); Coxsackie A, types 7 and 9, B, types 1 through 4 (mouse); influenza A, B and C (rooster); herpes simplex (guinea pig); distemper (canine); rubella (human), mumps (human), psittacosis (pigeon), and *Rickettsia burnetii* (bovine). Pooled normal Rhesus monkey sera were also employed. Neither neutralization nor delay in appearance of cytopathogenic effect occurred with any of the antisera when tested against 10^4 TCID₅₀ of 2060.

Convalescent sera from the recruit from whom 2060 was isolated did not demonstrate rises in neutralizing antibody titer to the adenoviruses, types 1 through 7. Additional indirect evidence against relationship to the adenovirus or Coxsackie viruses was obtained by means of the complement-fixation test. Rises in titer of complement-fixing antibody against adenoviruses, types 4 and 7 or Coxsackie A, types 7 and 9, and Coxsackie B, types 3 and 4, were not found. All testing was controlled with known positive sera.

Inoculation of tissue culture fluids containing 2060 into bacteriological media, *i.e.*, thioglycollate broth, fresh beef heart infusion broth with 20% horse serum, incubated both aerobically and anaerobically, beef heart infusion agar enriched with 10% sheep erythrocytes and 10% horse serum, incubated aerobically and under 10% carbon dioxide, failed to yield any indication of bacterial or mycotic growth after 14 days at 37°C. Further evidence against the bacterial

nature of the isolate was shown by the lack of effect of various antibiotic or chemotherapeutic compounds, including penicillin, dihydrostreptomycin, chloramphenicol, sodium sulfathiazole, sulfisoxazole diethanolamine and chlortetracycline, on the cytopathogenic effect of 2060 in monkey kidney cultures. Filterability of 2060 was demonstrated by passage of the isolate through a sintered glass filter (Corning, UF, pore diameter 0.9-1.4 μ).

Characteristics. After exposure of 10^4 TCID₅₀ to 70 or 95% ethanol, 1-800 para-tertiary - octyl - phenoxy - ethoxy - dimethylbenzyl ammonium chloride monohydrate and 5% phenol for 24 hours at 4°C, cytopathogenic effects were observed in monkey kidney cultures inoculated with a 10^{-1} dilution of the treated material. Controls without 2060 did not reveal specific effects, while those with the agent alone demonstrated typical changes at the same time. Similar treatment with 20% diethyl ether resulted in a delay in cytopathogenic effects. Even a pH range of 0.9 to 10.8, adjusted with HCl or NaOH, for 30 minutes at 4°C did not abolish the effects of 10^7 TCID₅₀; thus it appears that the agent was quite stable. As yet, a complement-fixing antigen has not been demonstrated despite repeated attempts with human and rabbit antisera and variations in amount of complement, hemolysin and time and temperature of fixation. Several methods were employed to induce agglutination of human type O, fowl, Rhesus monkey, sheep or rabbit erythrocytes. These included incubation at 4, 20 and 37°C, variation in pH from 5.3 to 10.1, dialysis of antigen and suspension of erythrocytes in 0.25M dextrose solution, dialysis and suspension in unbuffered 0.85% saline, heating the antigen at 70°C for 10 minutes, heating of the antigen followed by centrifugation at 12,000 x G for 30 minutes, and employment of erythrocytes treated with 0.1% trypsin for one hour. Hemagglutination with 2060 was demonstrated in titers of 64 to 128 with trypsin-treated human type O, fowl and monkey erythrocytes. Various proteins, including gelatin, inhibited this reaction, thus making it unsuitable for measurement of serum antibody.

Growth characteristics. Variations in the rates of increase of cytopathogenic effects depended upon the amount of the infecting agent (Fig. 2). A period as long as 2 to 3 weeks was necessary for complete destruction of cultures after inoculation with a minimal infecting dose. Because of the slow rate of growth, 10^3 or 10^4 TCID₅₀ as determined by cytopathogenic effects on the 10th day was used in neutralization tests to avoid non-specific changes found in older cultures. The fluid phase of the tissue culture also affected the development of cellular changes. There was a greater degree and more rapid appearance of cytopathogenic effects when mixture 199 was used without horse serum and bovine embryo extract. Similar cytopathology was obtained with bovine amniotic fluid medium, but such changes were not so pronounced when Scherer's MS, Eagle's(9) or 0.5% lactalbumin enzymatic hydrolysate media were used.

That 2060 was an infectious, propagable agent is suggested by an infectivity titer of 10^{-7} after the 13th passage of 0.1 ml aliquots in monkey kidney cultures containing 1.0 ml of fluid medium. Effects of an agent at this titer after such dilution are extremely unlikely in the absence of growth. Since the identity

TABLE I. Incidence of Neutralization Titer Increments* Associated with Respiratory Illnesses in Two Recruit Companies.

Type of illness†	Company A (Oct.-Dec. 1954)		Company B (Mar.-May 1955)	
	No. of cases	No. with titer rise	No. of cases	No. with titer rise
Cold	31	10	16	6
Diffuse	17	5	10	1
Pharyngitis	7	1	11	1
Chest	3	1	0	0

* 4-fold or greater increase in titer when tested with $10^{3.0}$ or $10^{4.0}$ TCID₅₀ of 2060 TC₁₃.

† Cold—coryza, afebrile; diffuse—sore throat, cough, coryza, with or without fever; pharyngitis—sore throat with dysphagia, with or without fever or tender cervical lymph nodes; chest—tracheitis, bronchitis, pneumonia, etc.

of early and late passage material was determined serologically, it appears that infectivity increments occurred in serial cultures.

Neutralization by human sera. Increments in titers of neutralization were demonstrated in human sera following respiratory illnesses as shown in Table I. These were observed in the recruit company from which 2060 was isolated and, also, in a second company studied during the spring of 1955. In all cases in which a similar type agent was isolated from nasal washings, at least an 8-fold increase in neutralization titer could be demonstrated within one month. The illnesses associated with titer increments were usually mild and of short duration, lasting from 2 to 5 days (to be published). Although these results indicated antigenic stimulation by the agent, probably due to infection, relation to illness may have been circumstantial. However, isolations as well as antibody rises occurred at times which suggested an etiological association. Further evidence of the extent of infection by 2060 was shown by neutralization titers of 1:32 in two lots of pooled human gamma globulin and by the demonstration of neutralization with sera obtained from children hospitalized in Chicago during the fall of 1955. From Fig. 3, it can be seen that despite irregularities in sampling, there was a tendency for higher titers to occur in sera of older children.

Discussion. That 2060 should be considered a virus is indicated by the results of

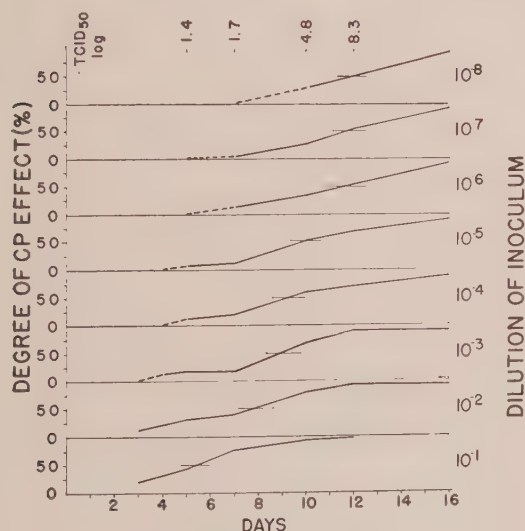


FIG. 2. Growth curves of logarithmic dilutions of 2060 based upon cytopathogenic effects in groups of four monkey kidney cultures. TCID₅₀ titers calculated from No. of positive cultures are shown at the time of each increment.

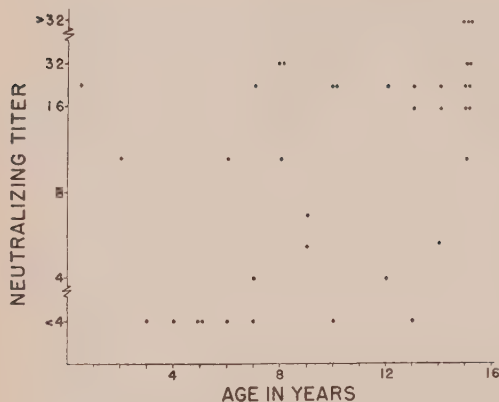


FIG. 3. Neutralization titers to 2060 in sera of children hospitalized in Chicago for a variety of medical and surgical illnesses.

these studies. Resistance to antibacterial agents, filterability, lack of growth on bacteriological media and absence of demonstrable organisms by microscopic examination of cells from tissue culture all support this contention. The lack of effect in suckling or adult mice, chick embryos, and HeLa cell cultures is characteristic of an unusual agent. When the slow rate of growth and type of cytopathogenic effect are included, the evidence favors classification of 2060 as a newly described virus or an atypical form of a known agent.

The lack of appearance of cytopathogenic effects prior to a second passage as well as increase in degree upon subsequent passages suggests adaptation to growth in monkey kidney cultures. Inhibitory substances in the nutrient medium in such cultures or in the nasal secretions, or minute quantities of virus in the original inoculum may also account for this observation. It is also of interest that certain synthetic maintenance solutions were not suitable for demonstration of cytopathogenic effects. Further studies are in progress to determine the significance of this observation and whether growth occurred in the absence of cytopathology.

That this was probably not a simian virus was indicated by lack of neutralization by at least 6 different monkey sera, complement-fixation with pooled monkey sera, or agglutination of monkey erythrocytes at 4°C as

has been reported with the majority of the simian agents(10).

Infection of humans probably occurred since increases in neutralizing capacity of sera were frequently found. A total of 13 additional cytopathogenic agents was obtained from nasal washings in the recruit company from which 2060 was isolated. Of these, 5 were neutralized by anti-2060 rabbit serum. Only one recruit from whom a 2060-type agent was isolated appeared asymptomatic. Serological evidence of infection in this man could not be determined because of unavailability of convalescent serum. An index of a widespread distribution was shown by neutralization with children's sera obtained in a different locality and year and by neutralization with human gamma globulin. Although at present the relationship of this agent to respiratory illness is only presumptive, artificial transmission of the disease to man as well as repeated isolations at the onset of respiratory illnesses, associated with serological evidence of infection will provide such information.

Summary. Several agents producing the same type of cytopathology have been isolated in monkey kidney cultures from men of a recruit company in training at Great Lakes in the fall of 1954. These isolates were obtained at the onset of mild respiratory infections and rises in neutralization titers to the prototype, 2060, followed each episode. Other increments in neutralizing antibody occurred in this and another company studied during the spring of 1955. Frequency of infection by 2060 was demonstrated by titers of neutralization found in children's sera from another locale and in pooled human gamma globulin. Characteristics of the isolate indicate that it is a relatively stable virus propagable in cultures of monkey kidney and testis and human embryo kidney with a slow rate of growth. Evidence has been presented which suggests that this agent may not have been previously described. The possibilities of etiological relationship to mild respiratory illnesses have also been discussed.

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Relation Between Degree of Fibrinogen Proteolysis by Plasmin and Fibrinolytic Paracoagulation.*† (22916)

S. SZUCHET AND M. DERECHIN.† (Introduced by R. Honorato)

Laboratory of Chemistry, Dental School, University of Chile.

Fibrinolytic paracoagulation, or clotting of lysed fibrinogen or fibrin(1,2), may be obtained only during a period which depends on experimental conditions. Previous works suggested a relation between degree of fibrinogen proteolysis and fibrinolytic paracoagulation which is here studied.

Materials and methods. Bovin fibrinogen (Fraction I from Armour Laboratories) was purified according to Laki(3) yielding preparations with 90-96% of total protein clottable by thrombin; chloroform activated plasmin was prepared by the method described by Loomis(4,5); Parke Davis thrombin with 5 mg/ml (approximately 100 u/ml) and 2M sodium sulphate (reagent grade) were used to effect paracoagulation, and phosphates buffer pH 7.6 to control pH. Reaction mixture: 1.5 ml of buffer and 7.5 ml of plasmin were added to 30 ml of fibrinogen solution and the mixture incubated at 40°C. Standard conditions throughout were: fibrinogen 5.150 g/l, pH 7.6 and ionic strength 0.2. Varying plasmin concentrations were employed in different reaction mixtures to reach final values of 0.60, 0.40 and 0.20 mg/ml respectively. Measurements of clotting time, paracoagulation test and non-protein nitrogen were made on ali-

quots withdrawn at suitable intervals. Determination of clotting time was carried out as follows: 0.9 ml of reaction mixture were transferred to test tube and 0.1 ml of thrombin added. The tube was shaken lightly and placed in water bath. The stop watch was clicked as thrombin was blown in and the time required for formation of a clot was recorded. When the mixture became unclottable paracoagulation was obtained with 0.5 ml of sodium sulphate forcibly blown into test tube, 1 minute after addition of thrombin. Paracoagulation was repeated until a negative test was obtained (extinction) and the time required for extinction was measured from initial addition of plasmin. To determine acid soluble split products, 4 ml aliquots of reaction mixture were discharged into equal amount of 10% trichloroacetic acid. The mixture was thoroughly shaken, allowed to stand 20 minutes and then filtered. Micro Kjeldahl digestions were carried out on 5 ml of filtrate using the catalyst mixture recommended by Chibnal, Rees and Williams(6) and N estimations were made according to Ma and Zuazaga(7). Acid soluble material at zero time was calculated from micro Kjeldahl estimations made on fibrinogen and plasmin specimens prior to their mixture. At least 4 curves for each plasmin concentration were carried out. Results are summarized in Fig. 1.

Results. As expected, the time required for the mixture to become unclottable by

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† We are indebted to Dr. J. B. Lesh from Armour Laboratories for supplying bovin fibrinogen.

‡ Research fellows of University of Chile (1955-1957).

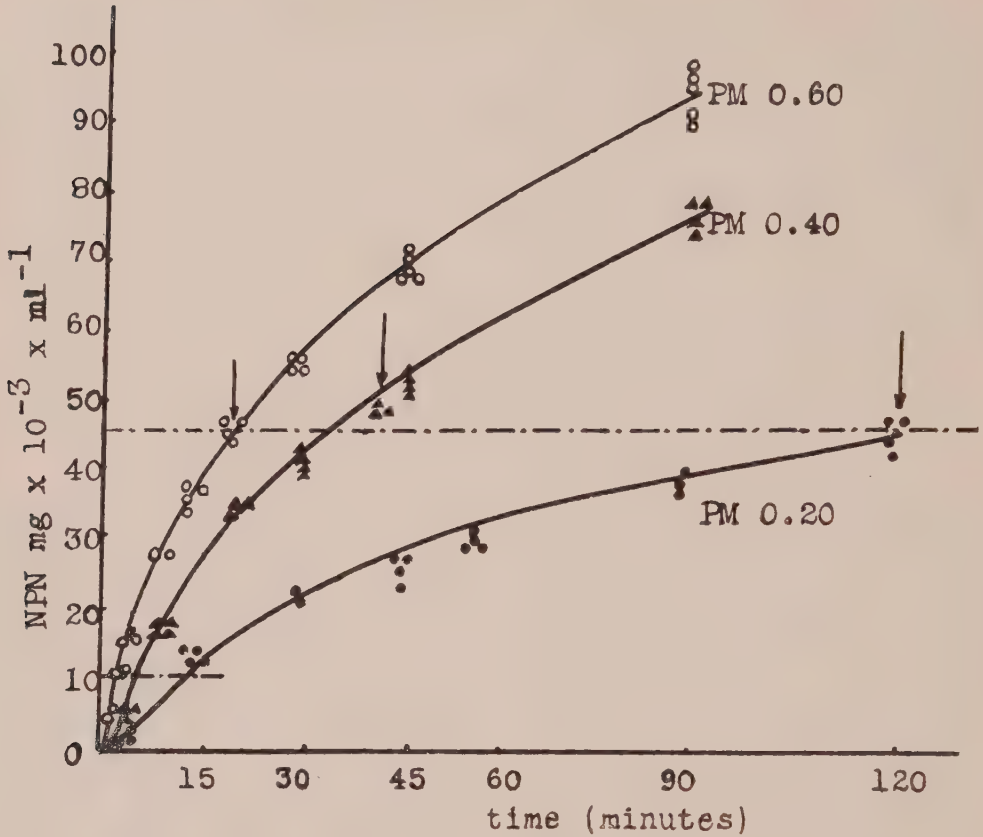


FIG. 1. NPN liberation and paracoagulation. Plasmin (PM) 0.60, 0.40 and 0.20 mg/ml of reaction mixture. Times for mixture to become unclottable by thrombin, 2½, 5 and 13 min., and for extinction of paracoagulation 20, 40 and 120 min. respectively. Arrows show experimental values when extinction was reached. An additional curve was obtained with saline instead of plasmin, but no increase in NPN was recorded.

thrombin and for extinction of paracoagulation differed for the 3 plasmin concentrations employed. Nevertheless, increase in NPN was the same for all curves at the time of extinction and amounted $45.7 \pm 0.54 \times 10^{-3}$ mg/ml of reaction mixture. NPN was not determined at time the mixture became unclottable by thrombin, but from interpolation data it was calculated to be nearly the same for the 3 series and amounted to approximately 10×10^{-3} mg/ml of reaction mixture. Though NPN liberation was mentioned by previous authors(8-10) who studied fibrinogen residues after lysis, it was neglected, and attention was drawn to the large residues. Holmberg(9) suggested that under the action of plasmin the fibrinogen molecule is split off into some few components whose molecular

weights from sedimentation and diffusion constants were calculated to be about 100,000. A small loss of N was attributed to possible attack on products of lysis by TCA acid. Kaplan(10) showed that the products of the action of plasmin on fibrinogen, which are non-coagulable by thrombin, could not be differentiated from fibrinogen and he suggested that the very small amounts of acid-soluble products which appear might result from a proteolytic activity of contaminant enzyme on fibrinogen.

The close relationship shown in our curves between levels of liberated NPN and behavior of lysed fibrinogen, when tested against thrombin and the paracoagulant substances, indicates that NPN liberated comes from plasmin-fibrinogen reaction, and that inten-

sity of paracoagulation is closely related to degree of fibrinogen proteolysis by plasmin. In this way, the small amount of acid-soluble material liberated is comparably important to the liberation of fibrinopeptides from fibrinogen by thrombin (3-4% of total N)(11), which gives rise to important changes in the original molecule. It is a matter of further study whether plasmin-liberated peptides play an active role as inhibitor or dissolution agents on the clotting of fibrinogen and on fibrin clot respectively.

Summary. Fibrinogen proteolysis by plasmin and the clotting of lysed fibrinogen were studied. Close relationship was found between increase in NPN and behavior of lysed fibrinogen.

We wish to express our thanks to Prof. R. Honorato for his guidance and encouragement.

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Comparative Metabolism of Ca⁴⁵ and Ra²²⁶* (22917)

B. J. STOVER, D. R. ATHERTON, AND J. S. ARNOLD

(Introduced by T. F. Dougherty)

Radiobiology Laboratory and Department of Anatomy, University of Utah, Salt Lake City.

In experimental biology, the double tracer technic is a simple but powerful method. It is especially useful for comparative studies of metabolically similar elements, since biological variability is greatly reduced. In the studies described below, the radioactive isotopes Ca⁴⁵ and Ra²²⁶ were used since the skeleton was of primary interest. The metabolism of radium, while qualitatively the same as that of calcium, quantitatively is unique, and the difference is of value in the study of skeletal metabolism. In addition, knowledge of the metabolism of radium relative to that of calcium is important in the problem of radium toxicity.

Methods. A 5-month-old beagle dog from a colony of several hundred(1) served as the subject. The pup was 1/2 to 3/4 expected maximum size, and its skeleton was still growing rather rapidly. 71.8 μ C Ra²²⁶ and

1.60 mc Ca⁴⁵ in 8.2 ml isotonic saline, pH ca. 2 (HCl), were administered. These amounts, which would not cause detectable radiation damage in a short term study, permitted the measurement of very small samples. The injection standard was measured with the same syringe subsequently used to inject the dog. A conventional proportional counter† was used to measure both Ca⁴⁵ and Ra²²⁶. Aliquots of ashed samples were plated on stainless steel discs, which were flamed to evolve radon and its daughters, then allowed to stand 1 to 2 hours to permit decay of any remaining radon daughters. Immediately afterward, alphas (from Ra²²⁶) were measured, then both alphas and betas (from Ca⁴⁵) were counted. The error in determining Ca⁴⁵ by difference was minimized by injecting 22.5 μ C Ca⁴⁵ per μ C Ra²²⁶. Measurement of both isotopes in the same sample eliminates the effect of preparation errors in

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† Nuclear Measurements Co., Model PC-1.

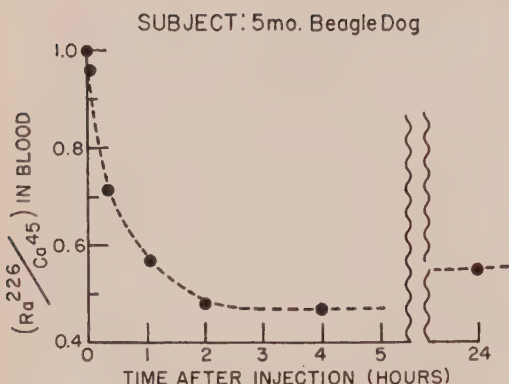


FIG. 1. Relative amounts of Ca⁴⁵ and Ra²²⁶ in blood at various times following intrav. inj.

determining relative amounts. Appropriate corrections for mass absorption were made, and aliquots of the injection standard were plated and measured with each group of samples. Blood samples were drawn at various times prior to sacrifice, which was at 24 hours after injection. Pre-sacrifice excreta and contents of bladder and gut were collected. The skeleton was completely dissected, and the teeth and individual bones were analyzed.

Results. A. Blood: Both alkaline earths serve as tracers for calcium; Ca⁴⁵ quantitatively, and Ra²²⁶, qualitatively. Early blood concentrations indicate the rates at which they are diluted by various metabolic processes involving calcium. The concentrations of both isotopes in blood were determined at times ranging from 1.4 min. to 24 hours after injection. Ca⁴⁵ is significantly greater than Ra²²⁶ at all times except at 1.4 min., when they are about equal. Thus, one or more of the calcium metabolic processes is significantly differentiating between the two alkaline earths. The ratio of Ra²²⁶ to Ca⁴⁵ in blood which is plotted against time in Fig. 1, decreases rapidly, attaining a minimum value of about 0.48 by 2 hours, which is maintained for several hours. The subsequent increase observed at 24 hours may be real, since additional studies(2) indicate two processes which could cause a slight increase. First, resorption occurring at the epiphyseal lines would release approximately equal amounts of the isotopes to the circulation. Second, Ca⁴⁵ is preferentially incorporated in mature

cortical bone, and, if this is a continuous process, it must be accompanied by a preferential release of Ra²²⁶ to the blood.

Assuming a blood volume of 430 ml, (*i.e.* 7% of body weight of 6.14 kg), the data can be expressed as % of injected dose of blood as a function of time. These data, which appear in Fig. 2, can be described by the following equations, each of which adequately describes all but the earliest point: % Ca⁴⁵ in the blood = $85t^{-0.70}$, % Ra²²⁶ in the blood = $63t^{-0.76}$, (t in min.). The method of least squares was used. (The first 20 minutes can be approximated with the functions % Ca⁴⁵ = $33t^{-0.42}$ and % Ra²²⁶ = $33t^{-0.54}$.)

Using the above equations, VPRC = 0.42, and serum calcium = 0.11 mg/ml, the average specific activity, ($\bar{SA}$) for both isotopes and several intervals has been calculated, and the results appear in Table I. Specific activities are expressed in units % dose/g calcium to facilitate comparison with bone specific activities. Also included are the products $\bar{SA} \times \Delta t$ normalized to the entire period, and ratios of Ra²²⁶ SA to Ca⁴⁵ SA.

B. Excreta. Results of excreta analyses are summarized in Table II, and show that both excretory routes are factors in the more rapid removal of Ra²²⁶ from blood. Although Ra²²⁶ excretion is approximately a factor of 10 greater than Ca⁴⁵ excretion, it is still only

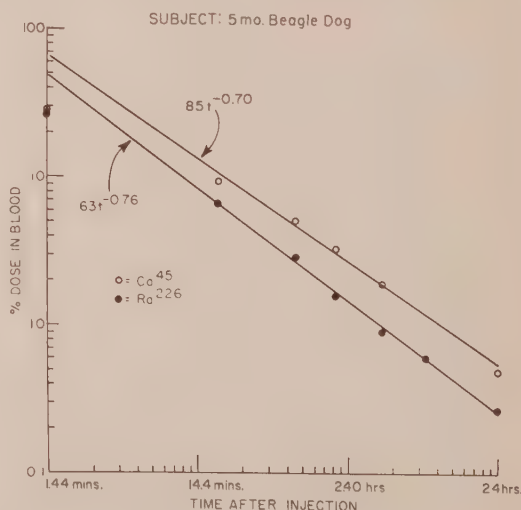


FIG. 2. % of inj. dose of Ca⁴⁵ and of Ra²²⁶ in blood following intrav. inj.

TABLE I. Average Blood Specific Activities of Ca⁴⁵ and Ra²²⁶.

Δt , min.	Ca ⁴⁵		Ra ²²⁶		$\frac{\text{Ra}^{226} \bar{SA}}{\text{Ca}^{45} \bar{SA}}$
	$\bar{SA}$, % dose/g Ca	Relative $\bar{SA} \times \Delta t$	$\bar{SA}$, % dose/g Ca	Relative $\bar{SA} \times \Delta t$	
0-20	585	.15	516	.23	.88
20-1440	46	.85	25	.77	.53
0-1440	54	1.00	32	1.00	.58

TABLE II. Results of Excreta Analyses.

	% of inj. dose		
	Ca ⁴⁵	Ra ²²⁶	Ra ²²⁶ /Ca ⁴⁵
Pre-sacrifice excreta	.26	2.85	11
Bladder & contents	.018	.25	14
Small intestine & contents	.14	.19	1.4
Large " " "	1.56	1.90	1.2
Blood at 24 hr	.49	.27	.55

a small fraction of the Ra²²⁶ injected. Since this was a young animal with a growing skeleton, retention of both alkaline earths is high.

The ratios of Ra²²⁶ to Ca⁴⁵ in urine and blood at 24 hours, show a preferential kidney excretion of radium by a factor of 26 over calcium. Putnam[†] found that radium in rat blood is 87% ultrafilterable, and the dog calcium value obtained by the same method is about 50% (3). Assuming in this case that the diffusible fraction of Ra²²⁶ is twice that of Ca⁴⁵, the observed value of 26 shows that resorption of calcium in the kidney of a young growing beagle is at least 10 times that of radium. The ratio of Ra²²⁶ to Ca⁴⁵ observed in both large and small intestines at autopsy is about twice that of blood, and probably results from the greater diffusibility of blood radium.

C. Skeleton and teeth. At autopsy the entire skeleton was dissected, several long bones were selected for other studies (2), and all others were analyzed individually. Since the radioactive isotopes deposit symmetrically in the skeleton, the special studies were done on one of a pair, and the other was analyzed. The total skeletal retention (including teeth) at 24 hours was found to be about 91% for Ra²²⁶ and about 94% for Ca⁴⁵, from which the Ra²²⁶/Ca⁴⁵ ratio for the whole skeleton is calculated to be 0.96. The mean of the Ra²²⁶/Ca⁴⁵ ratios for the individual bones

was 0.96, $\sigma = 0.075$. No significant difference between the Ra²²⁶/Ca⁴⁵ ratios of long bones and those of flat bones was observed.

In contrast with the high ratio of the skeleton, the Ra²²⁶/Ca⁴⁵ ratio observed in the teeth was 0.49, which is approximately equal to the minimum value observed in blood and is significantly lower than the average blood value for the entire period. Comparing the Ra²²⁶/Ca⁴⁵ ratios observed in whole bones, teeth, and blood, it is apparent that the rates and/or mechanisms of isotope incorporation differ in the skeleton and the teeth. The high ratio observed in the skeleton indicates some combination of the following: 1) rapid, irreversible deposition of both isotopes, 2) a higher Ra²²⁶/Ca⁴⁵ ratio in the extravascular fluids than in the blood (resulting from the larger fraction of diffusible Ra²²⁶), and 3) preferential binding of Ra²²⁶ on the crystal surfaces. The low ratio observed in teeth, which at this age can be assumed to be depositing dentine, definitely indicates a preferential incorporation of Ca⁴⁵. The ratio for teeth is too low to be accounted for by rate of fluid movement only, especially since the ratio in the extravascular fluid is probably higher than that in blood.

A process occurring in calcified structures which has been shown to discriminate against radium is recrystallization (2), and possibly in teeth recrystallization is relatively more important in the incorporation of blood calcium than is new crystal formation or ion exchange. If the formation of new crystals is slow compared with those of bone, sufficient time may elapse between the deposition of successive layers for appreciable equilibration to occur between surface and interior (recrystallization), and calcium would be preferentially incorporated.

Summary. The comparative metabolism of

[†] Personal communication.

TABLE III. Specific Activities at 24 Hr.

	% Ca^{45} /g Ca	% Ra^{226} /g Ca	$\text{Ra}^{226}/\text{Ca}^{45}$
Blood plasma	18	9.8	.55
Skeleton	2.0	1.9	.96
Teeth	.84	.41	.49

calcium and radium has been studied in a young beagle dog. Ra^{226} left the blood more rapidly than Ca^{45} ; the preferential excretion of radium by both kidney and gut is a factor in explaining the blood data, and also is the principal gross metabolic difference. The retention of both alkaline earths was greater

than 90%, consistent with the fact that the skeleton was rapidly growing. Analysis of individual bones revealed no significant difference in the relative deposition of the two isotopes, but the teeth showed a preferential incorporation of calcium.

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Effect of Glycerine on Toxicity of Isoniazid in Mice. (22918)

BENJAMIN PRESCOTT, GLADYS KAUFFMANN, WALTER D. JAMES

(Introduced by B. J. Olson)

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, N.I.H., P.H.S., Bethesda, Md.

Isoniazid (INH) continues in widespread use as a therapeutic agent against human tuberculosis; but dosage is limited by its toxicity. This toxicity has been reduced in laboratory animals by simultaneous administration of appropriate amounts of sodium glucuronate and glycine with the INH(1). Some other chemicals, certain amino and alpha-keto acids, have also been found to be effective in increasing the tolerated dose of INH (2). In addition several organic solvents have now been investigated. One of the most promising is glycerine which has long been recognized as an effective bactericidal agent. As early as 1869 Mueller(3) prepared cowpox vaccine in approximately 75% glycerine solution, and found its activity to be unimpaired whereas extraneous microorganisms were destroyed. A thorough investigation of the antiseptic and germicidal properties of glycerine was reported by Rosenau(4), who concluded that, while 10% by volume favored growth, 50% glycerine inhibited all bacteria studied—some 10 pathogens and a few saprophytes. Locatelli and Bowden(5) and Wood (6) found that sulfa drugs were more effective for topical and surgical application re-

spectively when dispersed in glycerine. Fisher(7) reported that the tuberculostatic activity of streptomycin was enhanced in Youman's medium containing 2% glycerine. Montestruc and Garcin(8) reported successful use of glycerine-suspended BCG to detect allergy in BCG-vaccinated individuals. From these studies it may be deduced that glycerine in suitable concentrations is useful as an adjuvant. Furthermore its low toxicity for human beings was observed by Johnson, Carlson, and Johnson(9) in a group of individuals given 110 g daily for 50 days with no adverse manifestations. However, to our knowledge evidence of its capacity to reduce the toxic action of chemotherapeutic agents in animals has not been presented.

The present study is an investigation of the effect of glycerine on the toxic action of INH in mice. The experiments include: (a) acute toxicity of glycerine solutions in DBA mice, (b) effect of glycerine on acute toxicity of INH in mice, (c) effect of repeated administration of various dosage combinations of INH and glycerine, (d) blood levels in mice following combined administrations, and (e) effect of glycerine on bacteriostatic activity

TABLE I. Acute Toxicity of Isoniazid in Glycerine Solutions.

Isoniazid, mg	Glycerine, % by vol	DBA mice		
		No.	No.	%
0	25	20	20	100
0	35	30	29	97
0	50	38	34	89
0	75	27	21	77
	undil.	30	17	57
4	0	20	20	100
4	25	20	19	95
4	35	20	19	95
4	50	20	19	95
4	75	20	15	75
6	0	20	0	0
6	15	20	9	45
6	20	20	13	65
6	25	20	18	90
6	35	118	113	95
6	50	19	13	68
6	75	20	14	70
7	25	20	10	50
7	35	50	36	72
7	50	69	46	67
7	75	20	11	52
8	0	17	0	0
8	25	20	8	40
8	35	74	28	37
8	40	20	15	75
8	50	146	126	86
8	75	50	28	56
10	25	20	0	0
10	35	60	10	17
10	50	29	10	34
10	75	114	55	48
10	undil.	20	0	0
15	50	20	1	5
15	75	40	6	15
20	50	20	0	0
20	75	21	0	0

Results. Acute Toxicity of Glycerine and INH-Glycerine Mixtures in Mice. A wide range of concentrations of INH in a series of glycerine solutions was tested by gavage in DBA mice. Table I shows the results of the control series without INH, and of combinations of 4 to 20 mg INH per 20-g mouse (200-1000 mg/kg), each tested with 2 or more concentrations of glycerine. It is evident that in the control significant numbers of mice succumbed when more than 50% glycerine was administered. Similarly with a sublethal dose of INH, 4 mg, the number of surviving animals closely followed the control series, even when a concentration of 75% glycerine was used. However, with a lethal dose of 6 mg INH, 35% glycerine permitted 95% of the animals to survive. This survival rate was confirmed in 6 replicate experiments with a total of 118 mice. The group given 8 mg INH demonstrated the endpoint, inasmuch as 50% glycerine concentration was needed to counteract the INH toxicity and this amount of glycerine *per se* was slightly toxic (11% mortality). Above this dose the toxicities of both the chemotherapeutic agent and the solvent were involved. This was evident in that with 10 mg INH in 75% glycerine only 48% of the mice survived. It would thus appear that in DBA mice a single dose of 6 mg INH in 35% glycerine, or as much as 8 mg INH in 50% glycerine was tolerated almost as well as the sublethal 4 mg dose of INH in water.

in vitro of INH on *Mycobacterium tuberculosis var. hominis*.

Materials. 1. *Isoniazid powder*—isonicotinyl hydrazide, obtained from Hoffmann-La Roche, Inc. The INH was dissolved in aqueous glycerine solution such that the desired concentration of each per 20-g mouse was contained in 0.5 ml volume for oral administration. 2. *Glycerine*, 95% reagent grade, Sp. gr. = 1.249. Two commercial products were used. Aqueous solutions of glycerine were prepared to give the desired concentration by volume; e.g., "25% solution" indicates 0.312 g/ml of pure glycerol. 3. *DBA inbred strain of mice* maintained at National Institutes of Health.

Effect of repeated administrations of INH in glycerine solutions. Two groups of experiments were then carried out to ascertain the mortality following repeated oral administrations of these optimal combinations of INH with glycerine. A. *Daily administration.* As seen in Table II, daily doses (except Sundays) of sublethal 4 mg amount of INH in glycerine and of mixtures containing 6, 8, and 10 mg INH in glycerine resulted in extended survival time. With the 10 mg dose evidently several resistant animals were included inasmuch as 83% of the 30 survived one dose, in contrast to the 48% survival rate shown in Table I. It was unexpected that the sublethal 4 mg dose in 35% glycerine

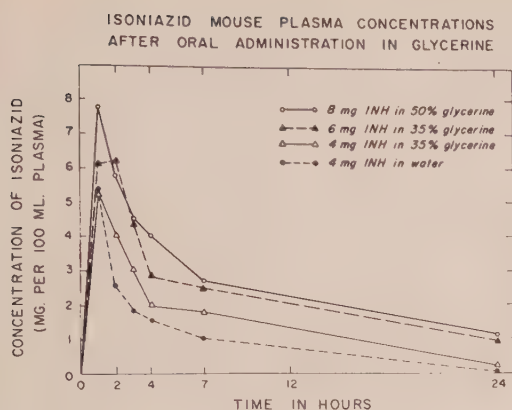


FIG. 1.

light of the promising results of the previous experiment on prolonged administration with this INH-glycerine combination. In addition, assay was made of the INH concentration in blood plasma from the mice sacrificed 2 hours and 24 hours after the 30th daily dose in the experiment described above. At the 2-hour interval, 3.1 mg% following administration of the aqueous solution is to be contrasted with the 4.3 mg% level following the glycerine solution. In the 24-hour bleeding of the latter group the INH persisted at 0.44 mg%. This is roughly comparable to the 24-hour level obtained when other INH detoxicants were tested(1) and somewhat higher than the 0.25 mg% of the control.

Effect of glycerine on in vitro inhibitory action of isoniazid on Mycobacterium tuberculosis. One procedure has been carried out to ascertain the effect of glycerine on the *in vitro* inhibitory action of INH on *Mycobacterium tuberculosis*. Following the technic described earlier(1), 2 human strains of tubercle bacilli have been used to determine INH inhibition endpoints with and without glycerine as solvent for the INH. The endpoints with INH alone were respectively: for strain H37Rv, 40 μ g/ml; and for strain 198 ARB, 5 μ g/ml. A 3% solution of glycerine as solvent did not change the INH inhibition endpoint with strain B103 (avirulent acid-fast). While there was questionable growth of this strain in control tubes containing 17.5% glycerine without INH, this glycerine concentration gave an endpoint for INH

at 2.5 μ g as contrasted with 10 μ g in the absence of any glycerine. But in the presence of 25% glycerine no growth of any of these strains developed with or without INH. In other words, there apparently is bacteriostatic synergy of the INH and glycerine at the higher concentrations.

Discussion. As is well known, glycerine in lower concentrations is a nutrient for bacteria. Pertinent to the present study, tubercle bacilli have been successfully grown on media containing from 2 to 12% glycerine(11). Long and Finner's studies 1927(12) showed that 7.5% to 10% glycerol gave maximum yield, as measured by dry weight of tubercle bacilli, with no growth at 15% concentration of glycerol. The *in vitro* results reported above also show that it is in the higher concentrations (25% and higher) that bacteriostatic effect is noted.

The long term *in vivo* experiment with 4 mg INH in 35% glycerine solution gave two unexpected results: first, that the animals were apparently in better physical condition than those receiving the aqueous solution of INH; and second, that they gained in weight normally in contrast to the retarded gain or even loss in the latter. It should be noted that some animals may have succumbed due to the mode of treatment, inasmuch as it has been observed that similar handling of normal mice to administer saline over the same prolonged period caused as much as 20 to 30% mortality.

While the effectiveness may be due in part to its ability to prevent the inhibition of certain enzymes by INH(13), it is possible that the action of the glycerine may be physical due to its hygroscopic characteristics and its viscosity, or some other mechanism as yet not explored.

Summary and conclusions. The toxic effect of isoniazid (INH) in DBA mice has been reduced by solution in several appropriate concentrations of glycerine. A sublethal dose of 4 mg INH (200 mg/kg) in 35% by volume of glycerine (10.9 g glycerol/kg) was administered orally 30 times within 36 days with a higher survival rate and fewer toxic manifestations than the same dose of INH

dissolved in water. Doses of 6 or 8 mg INH, lethal *per se* in aqueous solution, gave 50% mortality after 7 intermittent (twice weekly) administrations when 35% or 50% glycerine solution respectively was employed as solvent. With larger amounts of INH the requisite amount of glycerine lay in the toxic range. The absorption curves indicate that significantly higher blood plasma levels of INH were obtained with the glycerine solutions than with aqueous solutions. In higher concentrations of glycerine there was apparently synergism with INH in the *in vitro* bacteriostatic action on 2 human strains of *Mycobacterium tuberculosis*.

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Formation of Porphyrin from Delta-Aminolevulinic Acid by Uterine and Liver Tissue from Laying Hens.* (22919)

DONALD POLIN. (Introduced by H. Robinson)

Merck Institute for Therapeutic Research, Rahway, N. J.

A variety of avian tissues and organs are capable of forming porphyrins, *in vitro*, from delta-aminolevulinic acid (ALA); namely, chicken erythrocytes(1,2), duck erythrocytes (2,3), pigeon liver and breast muscle(4). The uterus of the hen has not been similarly characterized, and would be a likely organ to form porphyrin from ALA, *in vitro*, as oöporphyrin is deposited on the egg shell within this organ.

Methods and materials. White Leghorns, which lay white eggs, and Crossbreeds and New Hampshires, which lay brown eggs of varying shade, were used in these experiments. At a time when no egg was in the uterus, birds were killed by decapitation and uterus, portions of liver and of breast muscle were removed immediately and placed on crushed

ice. In a cold room (6°C), 1 g tissue was homogenized in a mortar containing coarse sand and 4 successive 5 ml aliquots of buffered saline solution, pH 7.2(5). After each grinding supernatant solution was decanted into a 25 ml graduate, and finally contents of the mortar were rinsed into the graduate with buffered-saline and diluted to 25 ml. The graduate was stoppered, the contents mixed and allowed to stand in crushed ice for 5-10 minutes. Aliquots of 0.2, 0.4, 0.8 and 1.6 ml (equivalent to 8, 16, 32 and 64 mg of tissue) from the upper half of the graduate were pipetted into tubes containing 1.4 ml of 0.008 M ALA in buffered-saline. One tube containing 1.6 ml of solution but no ALA, and another tube without the solution but with ALA were used as controls. All tubes were made up to 4.4 ml with buffered-saline, capped and incubated at 41°C for 24 hours. Each series of tubes was run in duplicate.

* Part of this paper was presented at Poultry Science Meeting, Aug., 1956.

After incubation, 5 ml of concentrated HCl were added to each tube. After 2 hours the contents were transferred to 15 ml conical centrifuge tubes with washings of buffered-saline. The volumes were made up to 12 cc with buffered-saline, and the tubes centrifuged. An aliquot of each tube was diluted with 3N HCl and read in the Beckman DU spectrophotometer against a control blank at 408 $m\mu$ and also at 382 and 432 $m\mu$ to obtain corrected density. Concentration of porphyrin was determined from a standard protoporphyrin curve. In experiments in which porphyrins were characterized, the incubated solution was first extracted with 4:1 ethyl acetate-glacial acetic acid until no further fluorescence could be observed in extracts examined under UV light. If porphyrins remained in the precipitate of the incubated solution, they were removed with 3N HCl washings. The acid washings were neutralized with saturated sodium acetate, extracted with ethyl acetate-acetic acid and added to porphyrin already obtained. Porphyrins were fractionated by the procedure of Dresel and Falk(2). Porphyrin was obtained from brown egg shells by dissolving the shells in 3N HCl and filtering the solution through Whatman #1 paper. The acid solution was adjusted to pH 4.8-5.5 with saturated sodium acetate, the oöporphyrin extracted with ethyl acetate-acetic acid, and fractionated(2). Absorption curves were determined on this oöporphyrin and on the porphyrins formed *in vitro* by supernatant solutions prepared from uterine tissues.

Results. Exp. 1. Supernatant preparations of liver and uterine tissues from hens laying brown eggs formed a considerable amount of porphyrins from ALA; whereas muscle preparations produced only trace amounts (Fig. 1). A linear relationship existed between amount of porphyrins formed and amount of tissue used, over the range studied. Regression equations (Fig. 1) were calculated from the data, and it was determined that 100 mg of uterine tissue, wet weight, formed 132 γ of porphyrins as compared to 62.5 γ by liver tissue (expressed in equivalents of protoporphyrin).

Exp. 2. Uterine and liver tissue prepara-

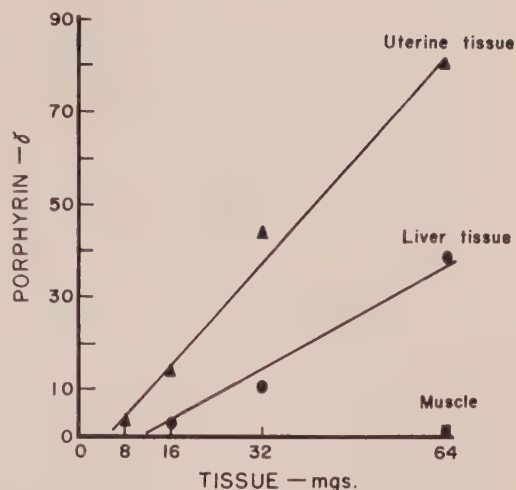


FIG. 1. Amount of porphyrin formed by uterine, liver and muscle tissues, from laying hens, incubated at 41°C for 24 hr in 4.4 ml buffered-saline, pH 7.2, containing .41 mg/ml of delta-aminolevulinic acid. $Y = -5.9 + 1.38X$ ($X = \text{mg uterine tissue}$); $Y = -8.3 + .708X$ ($X = \text{mg liver tissue}$).

tions from hens that laid white eggs formed as much porphyrin as similar tissue from hens that laid brown eggs (Tables I and II). There was considerable variation between hens in porphyrin-forming capacity of these tissues, but for each hen, there was a relatively constant ratio of approximately 2:1 for the amount of porphyrin formed per unit wet weight of uterine tissue as compared to liver. As shown in Table I, this ratio is approximately the same whether computed from ratio of slopes, $b_{\text{uterus}}/b_{\text{liver}}$ of regression equations or as a ratio of porphyrins formed per 100 mg tissue (obtained by extrapolation of regression equations).

TABLE I. Amount of Porphyrin Formed by Supernatant Preparations of Uterine and Liver Tissue from Hens Laying Brown or White Eggs.

Bird type and No.		Glandular tissue of uterus	γ porphyrins/100 mg tissue, wet wt—calculated from regression equations		Ratio of regression slopes
			Liver	Uterus	
Brown egg layer	1	132.8	62.5	2.12	1.97
	2	101.7	55.0	1.85	1.77
	3	105.2	53.8	1.96	1.88
White egg layer	1	116.8	61.6	1.90	1.74
	2	305.8	145.2	2.11	2.31

TABLE II. Porphyrin Forming Capacity of Supernatant Preparations of Uterine Tissue from Hens Laying Either a White, Tinted, or Brown Egg.

Brown egg layers		Tinted brown egg layers		White egg layers	
Breed	Porphyrin, $\gamma/100$ mg uterine tissue	Breed	Porphyrin, $\gamma/100$ mg uterine tissue	Breed	Porphyrin, $\gamma/100$ mg uterine tissue
New Hampshire	138.2	New Hampshire	105.2	White Leghorn	99.6
" "	132.8	Crossbreed	175.3	" "	429.0
" "	101.7	White Plymouth Rock	258.5	" "	260.8
" "	192.8	" "	271.9	" "	214.0

Exp. 3. Porphyrins formed *in vitro*, had an absorption curve similar to that of oöporphyrin (Fig. 2), but with maximum peak at 407-408 $m\mu$ and another at 480 $m\mu$. These porphyrins could be fractionated into 3 components tentatively identified as uro-, copro- and protoporphyrin. Uroporphyrin had an absorption peak at 405-407 $m\mu$ in 0.6N HCl, and comprised $91.4 \pm 1.4\%$ (mean $\pm$ S.E.M.) of porphyrins extracted. Coproporphyrin, with absorption peak at 401 $m\mu$ in .12N HCl, was present in trace amounts ($.5 \pm .18\%$), and protoporphyrin, with absorption peak at 407-408 $m\mu$ in 3N HCl accounted for $9.7 \pm 0.98\%$. These results are based upon analyses of tissue from 7 different hens.

Two to 3 g samples of uterine glandular tissue, from hens that laid brown eggs, were extracted for porphyrins, in the dark for 18 hours. The amount of porphyrins found in 5 different uteri ranged from 1.1 to 15.5 γ/g (mean = 6.9 γ/g) of uterine tissue. Partition of the porphyrins showed that a protoporphyrin-type compound (with the same

characteristics as shell porphyrin) comprised 97.1% of the total; whereas coproporphyrin accounted for the remainder.

Discussion. Investigators have suggested that shell porphyrins on the hen's egg are derived by hemoglobin degradation when erythrocytes are ruptured in the uterus, and pigment masses excreted onto shell(6). Although hemoglobin degradation to porphyrin cannot be excluded as a source for shell protoporphyrin, this pathway has not been proved. In fact, data indicate that hemoglobin degrades to bile pigments, *in vivo* (7), while protoporphyrin is formed via heme synthesis(8,9).

Porphyrins are synthesized in a cycle of which ALA is an intermediate(8,9). Uterine tissue contains considerable capacity for transforming ALA to porphyrins, *in vitro*, and it is suggested this tissue forms shell porphyrins via this cycle. The avian red cell can form protoporphyrin from ALA both *in vitro* (1,3) and *in vivo*(8,9), and may possibly contribute to the shell porphyrin pool in this manner, rather than by degradation of hemoglobin as suggested(7).

Most interesting is the fact that uterine tissue of a hen that laid a white egg, whose shell contained less than 10 γ of oöporphyrin, forms as much porphyrin, *in vitro*, from ALA as uterine tissue from a hen that laid a brown egg, the shell of which contained as much as 100 to 350 γ oöporphyrin. There is a possibility that ALA, or a precursor to ALA, is limiting in uterine tissue of the hen that lays a white egg, and this may account for the very small amount of oöporphyrin on the shell.

Sample preparations and substrate concentrations(2), in addition to other unknown factors, may account for dissimilarity in type

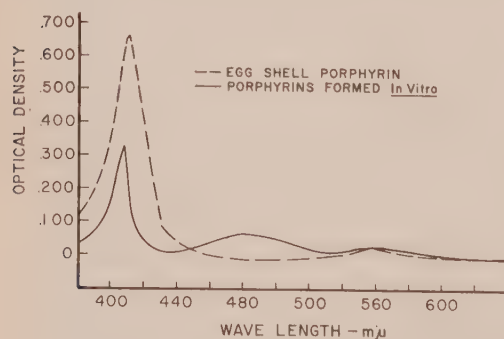


FIG. 2. Absorption spectra of porphyrin from egg shell (max. 410 $m\mu$) and porphyrins formed by uterine tissue supernatants (max. 408 $m\mu$), both in 3N HCl. Measurements were made with a Beckman DU spectrophotometer. Cell thickness, 1 cm.

of porphyrins formed *in vitro* as compared to those formed *in vivo*. Uroporphyrin-like compounds are formed predominately by preparations of uterine tissue; whereas, almost all porphyrin deposited on shell and found in intact uterine tissue is a protoporphyrin-type compound. Some coproporphyrin, 0.2 γ /g of uterine tissue, is also found.

Summary. 1. Uterine tissue, from white or brown egg-laying hens, forms twice as much porphyrin, *in vitro*, from delta-aminolevulinic acid as liver tissue. Supernatant preparations of uterine tissue form predominately uroporphyrin, small amounts of protoporphyrin, and trace amounts of coproporphyrin from delta-aminolevulinic acid. These compounds were tentatively identified by their behavior in the fractionation procedure used and by their absorption curves. 2. Oöporphyrin and coproporphyrin were extracted from the glandular tissue of the uterus of lay-

ing hens.

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Role of Lipid in Hemagglutination by *Candida albicans* and *Saccharomyces cerevisiae*.^{*} (22920)

RALPH A. VOGEL. (Introduced by J. H. Peters)

Research Laboratories, Veterans Administration Hospital, Atlanta, Ga. and Department of Bacteriology, Emory University School of Medicine.

An erythrocyte sensitizing antigen (ESS) from saline washings of bead-disrupted *Candida albicans* and *Saccharomyces cerevisiae* has recently been described(1). The activity of the antigen was measured in the hemagglutination reaction in which simple admixture of erythrocytes with polysaccharides leads to acquisition of immunologic activity by the red cells as demonstrated by their agglutination in homologous antiserum. In addition to such activity these preparations were noted to possess immunologic precipitating properties while on the basis of positive Molisch

tests and negative Biuret tests they were characterized essentially as polysaccharide. Recent observations have shown that occasionally the erythrocyte sensitizing activity of an antigen may disappear spontaneously on standing while its precipitating activity remains unchanged.

In the experiments reported below it is proposed that saline extracts from these organisms, with the overall properties of polysaccharides, contain lipid which is necessary for the sensitization of red cells. The current interest in the pyrogenic, endotoxic and hemagglutinative properties of the lipopolysaccharides from Gram-negative bacteria(2,3) makes the findings reported below of antigenic compounds believed to be lipopolysaccharide isolated from yeast and yeast-like organisms of considerable interest.

Materials and methods. Antigen prepara-

^{*} The author is indebted to Mary E. Collins for technical assistance and to Dr. Morris Tager, Chairman, and Dr. Frank Lanni, Emory University School of Medicine, for many helpful suggestions.

Pancreatic extract, lot # 8279-3 was kindly supplied by Dr. Kenneth W. Thompson, Medical Director, Organon Inc.

tions: a. *Solid medium*. *C. albicans* or *S. cerevisiae* were cultured on Sabouraud's dextrose agar in Roux bottles. The antigen was harvested with isotonic saline and extracted as described in a previous report(1). b. A liquid medium containing 4% dextrose and 1% neopeptone was distributed in gallon jugs and aerated after inoculation with 0.3% by volume of either *C. albicans* or *S. cerevisiae*. Cohen's filtration method(4) was used to separate cells from medium after 48-72 hours incubation. The antigen, produced free in the surrounding medium, was concentrated approximately 30 times by pervaporation or ultrafiltration. It was adjusted to contain 10% sodium acetate, 1% acetic acid and then treated with 2 vol. of 95% alcohol. The resulting precipitate was centrifuged and reconstituted in isotonic saline. *Antisera and immunologic tests*. Cells from liquid cultures were used to immunize white rabbits with either *C. albicans* or *S. cerevisiae* according to an immunization schedule given elsewhere (1). A qualitative precipitin test in which antigen dilutions are layered over undiluted antisera in 6 mm tubes was employed to estimate comparative strength of antigen preparations. A quantitative precipitin test was also employed essentially as described by Kabat and Mayer(5). Antibody nitrogen was determined by a modified biuret technic(1). The hemagglutination test described in a previous report was used to estimate and measure the sensitizing capacity of the antigen preparations(1).

Results. Antigens derived from both liquid and solid mediums exhibited precipitin activity and showed the presence of ESS as measured in the hemagglutination reaction. The antigens differed in appearance and serologic activity. The liquid antigen gave positive ring tests in homologous antiserum in dilutions of 1:1080 as opposed to 1:200 for the solid antigen. However, a 1:8 dilution of solid antigen as opposed to a 1:2 for the liquid antigen was capable of sensitizing red cells in the hemagglutination test. The lack of correlation between the precipitin titers and ESS activity of these antigens is noteworthy.

TABLE I. Removal of ESS from *C. albicans* Antigen by Seitz Filtration and Exhaustive Deproteinization.

Procedure done on antigen	Precipitin test (titer)*		Hemagglutination test (titer)*	
	Before	After	Before	After
Seitz filtration†	1:200	1:800	1:1080	0
Deproteinization‡	1:1080	1:2160	1:128	0

* Precipitin test: highest dilution of antigen giving a positive ring test. Hemagglutination test: highest dilution of antiserum causing hemagglutination of red cells.

† Antigen diluted 1:4 before Seitz and tested undiluted after Seitz.

‡ Antigen concentrated 2-fold after deproteinization.

Removal of ESS by deproteinization procedures. Exhaustive treatment, 6-7 times, of the antigens from either *C. albicans* or *S. cerevisiae* with a chloroform and n-butyl alcohol mixture as required in Sevag's deproteinization technic(6) led to removal of ESS and residual protein but no loss of precipitating activity (Table I). It has not yet been possible to determine whether the ESS is removed at the emulsion interface layer or in the chloroform layer. The possibility of the ESS being protein is slight since Boyden(7) has shown that erythrocytes must be chemically pretreated before they will adsorb proteins preferentially over polysaccharides.

Removal of ESS by Seitz filtration. The ESS was removed from *Candida* or *Saccharomyces* antigen preparations when small aliquots were passed through Seitz filters and, as

TABLE II. Quantitative Nitrogen Determinations on Antibody Precipitated from *Saccharomyces* Rabbit Antiserum by Seitz Filtered *Saccharomyces* Liquid Antigen.

Amt of antigen (ml)	Antibody N (mg)/ml of antiserum			
	Before Seitz (dil. 1:4)		After Seitz (undil.)	
	(1)*	(2)	(1)	(2)
.02	.064	.063	.112	.113
.05	.160	.144	.184	.192
.10	.192	.244	.208	.280
.20	.208	.304	.209	.304
.30	.208	.304	.184	.280
.40	.184	.280	.160	.256
.50	.160	.259	.160	.256
.60	.160		.144	

* (1) and (2) represent individual tests using different antisera and antigen samples.

TABLE III. Inactivation of ESS from *C. albicans* and *S. cerevisiae* by Lipase as Measured in Hemagglutination Test.

Red blood cells sensitized by <i>C. albicans</i> antigen after treatment of latter with	Titer of <i>C. albicans</i> antiserum causing hemagglutination	Red blood cells sensitized by <i>S. cerevisiae</i> antigen after treatment of latter with	Titer of <i>S. cerevisiae</i> antiserum causing hemagglutination
Lipase (0.6 mg/ml) for 0 hr at 37°C	1:4096	Lipase (0.16 mg/ml) for 0 hr at 37°C	1:4096
5 37	1:1024	5 37	1: 512
25 37	1: 512	24 37	0
72 37	0	24 70	1: 64
72 70	1:2048	NaCl (control) 24 70	1: 64
NaCl (control) 72 37	1:4096	24 37	1:4096
72 70	1:4096		

noted with deproteinization, no loss was observed in precipitin activity (Table I). As can be seen in Table II, precipitin properties were not quantitatively diminished by Seitz filtration. Furthermore, absorption of either *Candida* or *Saccharomyces* antiserum with Seitz-filtered, ESS-negative antigens completely removed antibody agglutinating red cells sensitized with the unfiltered antigens.

Blocking of ESS by addition of albumin. Neter(8) showed that various protein substances inactivate the ESS from *E. coli*. The blocking action of the protein is against the antigen itself and not the red cells. Bovine serum albumin, Fraction V, in 0.2% concentration inactivated the ESS of both *Candida* and *Saccharomyces* antigens. Egg albumin was less active but this may be attributable to poorer solubility of egg albumin in saline. Inactivation of the antigen again was not accompanied by a reduction in precipitin activity.

Inactivation of ESS from C. albicans or S. cerevisiae by lipase. The following experiments with lipase indicate that lipid is in some way involved in sensitization of red blood cells. A purified, defatted pancreas preparation was employed. Large quantities of both *Saccharomyces* and *Candida* antigens were passed through Seitz filters to assure sterility. This procedure did not affect antigen activity as noted previously when small aliquots were filtered. Pancreatic lipase powder was added to the antigens in concentrations ranging from 0.6 mg per ml to 0.16 mg/ml. Merthiolate was added in a final concentration of 1:50,000. Samples of anti-

gens plus lipase were incubated at 37°C and 70°C, and tested after various intervals for their ability to sensitize red cells.

The results of these tests are given in Table III. Both *Candida* and *Saccharomyces* antigens were inactivated by pancreatic lipase. If lipase was added to the antigens before Seitz filtration there was no loss in antigen activity. Likewise no loss, due to lipase activity, was noted if the lipase-antigen mixture was kept at 70°C. Since lipase is inactivated at temperatures above 55°C, and also if passed through a Seitz filter, these data would indicate that lipase acts enzymatically to destroy the sensitizing properties of the antigens. *Saccharomyces* antigen appeared to be a more suitable substrate for lipase and more heat-labile than the *Candida* antigen.

Discussion. Keogh, North and Warburton (9) isolated erythrocyte sensitizing antigens from many Gram negative and positive organisms. In their early work they hypothesized that type-specific polysaccharides isolated from these organisms apparently required a second compound in order to adsorb the former on to the surface of red cells. They later felt that polysaccharide antigens which failed to sensitize had undergone chemical degradation and there was no need to postulate a second substance. It was felt by the present investigator that if polysaccharides alone were responsible for modification of the red cell surface in the hemagglutination test, the potential strength of antigens doing this should be predicted by their comparative activity in the qualitative precipitin test, in which polysaccharides readily give positive re-

actions. During the present work with polysaccharides from yeast and yeast-like organisms which were extracted and purified by gentle procedures, or treated as described in this report, it was consistently noted that precipitin activity of the antigens did not parallel their ability to sensitize red cells. It was further noted that there was no difference in quantitative precipitating activity of antigens which are active erythrocyte sensitizers at one stage of handling and then inactivated after purification or other manipulations described in this report. The inactivation of the ESS activity by lipase or by deproteinization with organic solvents would seem to implicate a lipid containing antigen in sensitization of red cells.

Role of lipids in erythrocyte sensitization. In recent months groups of investigators led by Neter(2) and Landy(3) have isolated antigenic lipopolysaccharides from *E. coli* and *S. typhosa* respectively. These materials extracted by Boivin's trichloroacetic acid method or by the phenol/water extraction method of Westphal and associates(10), exhibit erythrocyte sensitizing activity, pyrogenic and endotoxic properties. Both have observed that heat or mild alkali treatment of their antigens is necessary before they will sensitize erythrocytes. The release of lipid by this treatment is believed by Landy to have something to do with the activation of the material (2). However, attempts to block the active antigen with the released lipid have been unsuccessful. It is interesting to note that total bound lipids are not removed by heat or alkali but require mineral acid hydrolysis. Unlike the findings of the authors cited, neither heat treatment nor alkali is necessary to activate erythrocyte sensitizing properties of the yeast antigen by removing lipid. On the contrary a lipid fraction appears to be essential for sensitization of the erythrocytes.

Neter *et al.*(11) have shown that lecithin and cholesterol inactivate the *E. coli* antigen when used in extremely small amounts. The inactivation effect is enhanced by boiling the lipid-antigen mixtures. The pancreatic extract used in the present report contained 0.5 mg of cholesterol per gram. However, experiments conducted with pure cholesterol

showed no inactivating effect on the yeast antigens when used at concentrations considerably higher than that found in the pancreatic extract. Landy reports(3) that using the enzyme pancreatin in an attempt to hydrolyze bound lipid from his antigen he succeeded in activating its erythrocyte sensitizing properties. However, it was found that activation was not due to enzyme activity but to alkali buffering the enzyme-system.

Lipopolysaccharide-protein complexes. Neter(2) described the ability of the lipopolysaccharides from *E. coli* to complex with protein. These complexes are only stable, *i.e.* not precipitated with trichloroacetic acid if the antigen is first treated with alkali or heat. The antigens resembling lipopolysaccharides from yeast or yeast-like organisms also show an affinity for complexing with protein in this highly stable manner without requiring preliminary treatment.

Further investigations are in progress to determine the exact chemical nature of the yeast-like antigens responsible for sensitizing erythrocytes, and to explore the possibility that these fungi contain materials similar to the lipopolysaccharides of Gram negative organisms which are pyrogenic and endotoxic.

Summary. The erythrocyte sensitizing antigen in saline extracts from yeast and yeast-like organisms was inactivated by a number of procedures including exhaustive deproteinization with chloroform-n-butyl alcohol mixtures, Seitz filtration, albumin blocking and treatment with lipase. The erythrocyte sensitizing antigen from *S. cerevisiae* was found to be more heat-labile and more readily inactivated by lipase than the *C. albicans* antigen.

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Reticulocytosis Induced by Serum from Hypoxic Animals.* (22921)

DANIEL F. GRAY AND ALLAN J. ERSLEV. (Introduced by W. B. Castle)

Thorndike Memorial Laboratory and 2nd and 4th (Harvard) Medical Services, Boston City Hospital, and Department of Medicine, Harvard Medical School, Boston, Mass.

It has been demonstrated that whole plasma and serum from animals with blood loss anemia will induce a reticulocytosis when injected into normal animals(1-5). When large amounts of such plasma are injected over a prolonged period of time, increases in number of red blood cells, in hematocrit and in bone marrow normoblasts have been observed(3). The following experiment was carried out to investigate whether the serum of animals exposed to low oxygen tension of inspired air would induce a similar erythropoietic response.

Methods. To utilize the bioassay technic previously described(3) white New Zealand rabbits, weighing 2-3 kg, were used both as donors and recipients. Normal serum was obtained from normal animals kept at room air, and "hypoxic serum" from normal animals kept in 10% oxygen for 48 hours. The latter animals were kept in a bank of 8 cages, enclosed in an air tight envelope of plastic yard goods sealed around inlet and outflow tubes. Sufficient food and water was placed in the cages to provide for the animals until the end of the hypoxic period. Trays of calcium hydroxide and calcium chloride were used to absorb excess carbon dioxide and water. The air within the envelope was circulated by a small electric fan. Its temperature was checked and never rose above 31°C. The oxygen level in the envelope was kept constant at 10% by admitting compressed room air and pure nitrogen at appropriate

flow rates. The total mixed air flow was 0.75-1.0 liter per minute per animal. The actual oxygen level was checked frequently by use of Beckman electromagnetic oxygen analyzer. Four 48 hour runs were made with above equipment with 8-12 animals in each run. After each run 2 animals were kept alive in room air as "tracers." The others were bled to death by intracardiac puncture within 1½ hours after end of hypoxic exposure. The "hypoxic serum" so obtained was pooled and frozen or refrigerated until used. Normal serum was obtained and stored in a similar fashion. Normal recipient rabbits each received 50 ml of serum i.v. once a day for 4 days. Daily hemoglobin determinations and reticulocyte counts were carried out on recipient animals and on tracer animals from the hypoxic group of donors. Reticulocyte counts were made on cover glass smears pre-stained with brilliant cresyl blue and counter-stained with Wright's stain. Reticulocyte smears were counted "blind" and a minimum of 1000 red cells was examined—except on days 2, 3, 4, 5 when a minimum of 2000 cells was examined.

Results. Tracer animals. (Fig. 1). Eight rabbits, 2 from each of 4 runs, were followed after exposure to 10% oxygen for 48 hours. Although there was a wide variation in response, the mean reticulocyte level rose from a base line of 1.9% to maximum of 6.7% on day 3. Hemoglobin levels remained unchanged. **Normal recipients of normal serum.** (Fig. 2). Six normal rabbits received 200 ml of serum from normal donors. The reticulocyte levels rose slightly from a mean base

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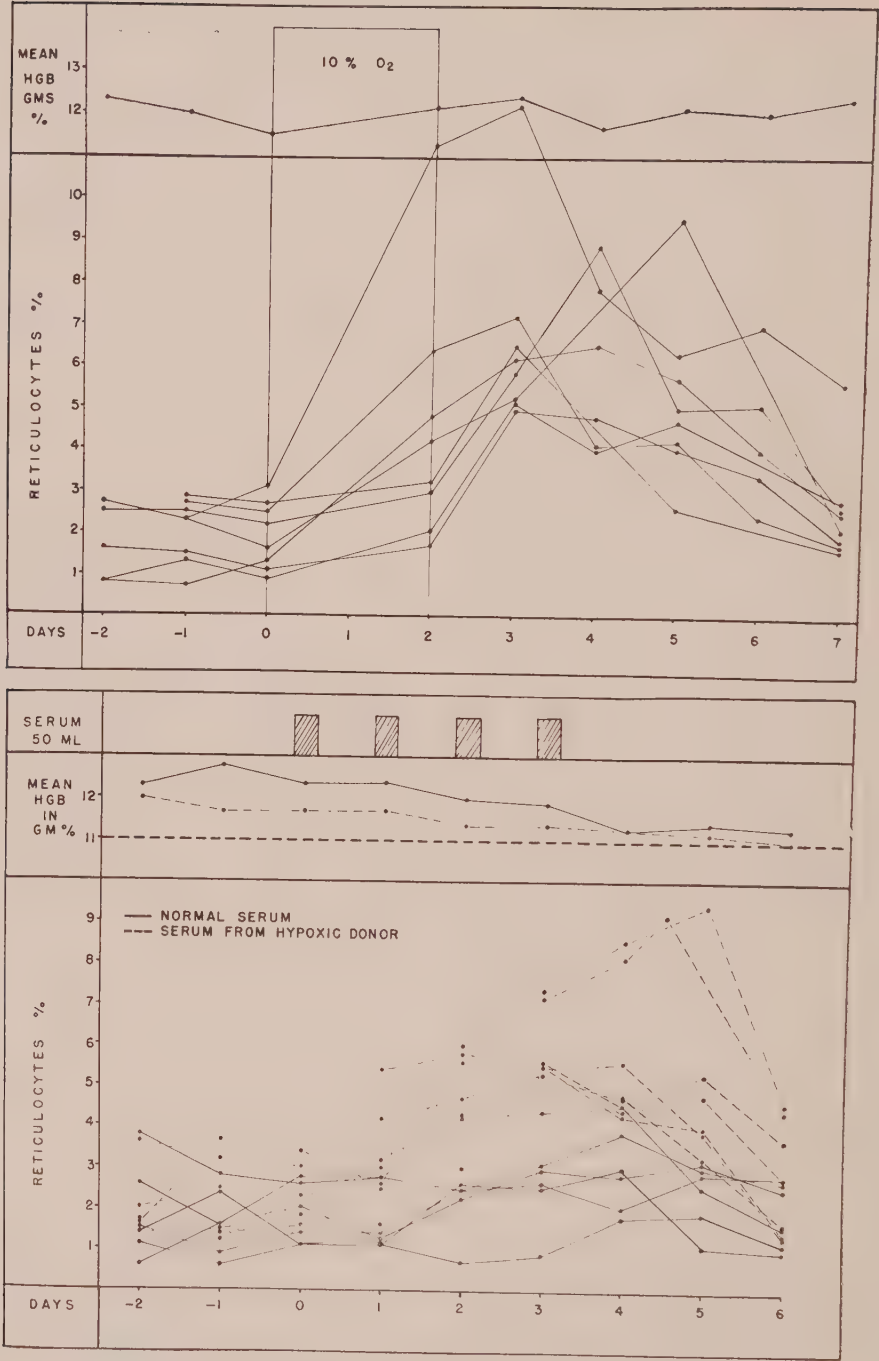


FIG. 1 (Top). Reticulocyte counts and hemoglobin concentrations of 8 rabbits exposed to 10% oxygen for 48 hours.

FIG. 2 (Bottom). Reticulocyte counts and hemoglobin concentration of six normal rabbits each receiving a total of 200 ml of serum from normal donors (solid lines) and of 7 normal rabbits each receiving a total of 200 ml of serum from donors exposed to 10% oxygen for 48 hours (stippled lines).

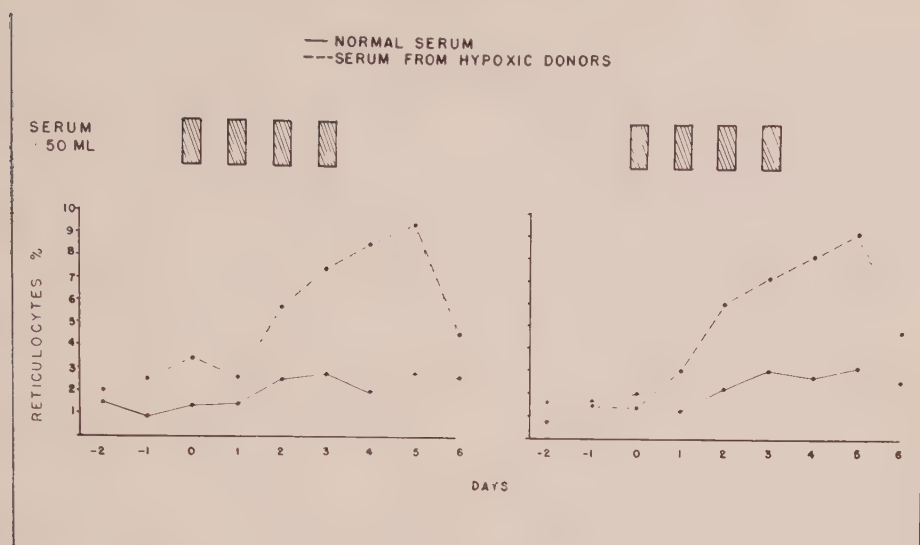


FIG. 3. Reticulocyte counts of 2 rabbits each receiving first 200 ml of "hypoxic" serum (stippled lines) and then, 2 weeks later, 200 ml of normal serum (solid lines).

line value of 1.9% to a maximum of 3.0% on day 4. Hemoglobin levels were essentially unchanged.

Normal recipients of hypoxic serum: (Fig. 2). Seven normal rabbits received 200 ml of serum from hypoxic donors. The mean reticulocyte counts rose from a base line level of 2.2% to maximum of 5.8% on day 4. Hemoglobin levels were essentially unchanged. Reticulocyte responses on day 2-5 differed from responses to normal serum in a statistically significant manner with a p value of less than 0.01.

Due to wide individual variation in recipients receiving the same serum, it was decided to use as recipients for normal serum the 2 animals which had the highest response to hypoxic serum. The normal serum was injected one week after the reticulocyte counts had returned to normal base line values. The results are shown in Fig. 3. There was no rise in reticulocyte levels during the second series of injections.

Discussion. A number of previous studies (see review by Grant and Root) (6) have indicated that serum from animals kept at low oxygen tensions is capable of inducing an erythropoietic reaction when infused into normal recipients. However, serious doubts have been raised as to the validity of the bio-

assay technics used (7) and a re-examination of this problem was felt to be justified.

The studies reported here do strongly suggest that serum of rabbits exposed to 10% oxygen for 48 hours contains a factor capable of stimulating erythropoietic activity in normal rabbits. Similar results in rats have been reported by Stohlman and Brecher (8). It is conceivable that the reticulocytosis seen in this study represents not an increase in red cell production, but a non-specific release of preformed reticulocytes from bone marrow. However, the response is very similar to that seen in tracer animals after exposure to 10% oxygen for 48 hours, and also to the reticulocytosis induced by serum from animals made anemic by bleeding. Both of these responses have been shown to reflect true erythropoietic activity.

A surprising finding was that the reticulocyte responses of the rabbits to hypoxia itself, and to "hypoxic serum" were essentially the same. At first glance it would seem hard to understand why the recipients should have as great and in some cases greater response than animals exposed directly to low oxygen. Certainly, the concentration of any factor would be higher in the donor than in the recipients, where it is diluted immediately 1:3 in the plasma volume. The explanation for this

phenomenon is perhaps, that although the stimulating factor or factors were necessarily diluted in the recipients' plasma, they exerted their effect over a longer period of time, causing a sustained and additive stimulation.

Conclusions and summary. 1. Once a day for 4 days 7 normal rabbits each received 50 ml of serum obtained from hypoxic rabbits, and 6 normal rabbits each received the same amount of serum obtained from normal rabbits. The hypoxic serum induced a reticulocytosis which was significantly different (p value < 0.01) from the response induced by injection of normal serum. At the level of hypoxia used (10% oxygen for 48 hours) the response in the recipient animals was as great as that seen in the animals directly exposed to hypoxia. 2. These results bolster the hypothesis that the erythropoietic response to

hypoxia, or to blood loss anemia involves the production of a humoral factor.

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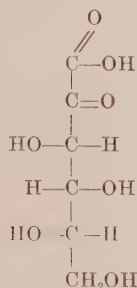
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Fate of 2-Keto-L-Gulonic Acid in Rat and Guinea Pig.* (22922)

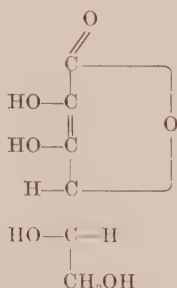
PETER G. DAYTON. (Introduced by J. Murray Steele)

Research Service Third (N. Y. University) Medical Division, Goldwater Memorial Hospital, Welfare Island, New York City.

The close structural relation between 2-keto-L-gulonic acid and L-ascorbic acid has prompted studies of 2-keto-L-gulonic acid as a possible precursor of L-ascorbic acid(1-6).



2-keto-L-gulonic acid



L-ascorbic acid

The recent finding that L-gulonolactone can be transformed by the rat to L-ascorbic acid(6,7) suggests that 2-keto-L-gulonic acid could be an intermediate in this transforma-

tion. For these reasons experiments were carried out in guinea pigs and rats with uniformly C^{14} labeled 2-keto-L-gulonic acid. The results of this study indicate no appreciable conversion of this compound to L-ascorbic acid in either species.

Methods. 2-Keto-L-gulonic acid C_6^{14} was synthesized from L-sorbose C_6^{14} according to modification of the method of Reichstein and Grüssner(8-11). The compound had a specific activity of $0.2 \mu\text{c}/\text{mg}$ and melted at 171°C . Titration of a 5 mg sample of the labeled substance with indophenol dye(12) showed that no more than 0.25% of L-ascorbic acid could be present. Radioactive purity was established by 2 criteria. The first consisted of finding no change of activity upon recrystallization with carrier from a solvent mixture consisting of methanol, acetone and ligroin. Secondly, L-ascorbic acid prepared from the labeled compound(10) had the same molar specific activity when counted as

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its 2,4-dinitrophenyl-osazone derivative(13). Male Wistar rats, weighing 275-325 g, were given a diet of milk for 5 days prior to and during each experiment. Guinea pigs obtained from Rockland Farms and weighing about 300 g were maintained on a Vit. C-free diet supplemented by 5 mg of L-ascorbic acid for 5 days before the experiment. The labeled compound was dissolved in 1-3 ml H₂O and injected intraperitoneally. Determination of C¹⁴ in respiratory CO₂ and urine was carried out as previously reported(14). The amount of C¹⁴ present in urine as unchanged 2-keto-L-gulonic acid was determined as follows: The urine was collected during 24 hours after the dose in the presence of 1500 mg of carrier 2-keto-L-gulonic acid[†] and diluted to 200 ml. The 2-keto-L-gulonic acid was then transformed to L-ascorbic acid by a modification of the method of Reichstein and Grüssner(10). To a 100 ml portion 3 ml 6N HCl were added and it was refluxed under N₂ for 2 hours at 100°C. The solution was then quickly cooled to room temperature. Titration of an aliquot with indophenol dye, indicated a yield of L-ascorbic acid of about 20%. The L-ascorbic acid was oxidized to dehydro-L-ascorbic acid with bromine water; excess bromine was removed by flushing the solution with N₂. The solution was then passed through a column of Amberlite IR-4B resin in the acetate form (a resin bed 15 cm long and 2.5 cm in diameter was found satisfactory). Dehydro-L-ascorbic acid being neutral passed through the column, whereas 2-keto-L-gulonic acid was adsorbed. The dehydro-L-ascorbic acid in the effluent was converted to its 2,4-dinitrophenylosazone derivative(13) which was crystallized from 1:1 acetone-ethanol. The derivative melted at 285-287°C (mixed melting point undepressed with reference compound) and its specific activity remained constant upon recrystallization. The conversion of 2-keto-L-gulonic acid to L-ascorbic acid by rats and guinea pigs was determined by a carrier dilution technic (7). In brief, the method involved determin-

TABLE I. Distribution of C¹⁴ 24 Hr after Intraperitoneal Administration of 2-Keto-L-Gulonic Acid C₆¹⁴.

Animal	Wt (g)	Dose (mg)	% of admin. C ¹⁴ found in		
			Respiratory CO ₂	Urine	L-ascorbic acid*
Rat	275	4.00	11	78	—
"	325	18.00	7.9	80	<1.2
"	310	18.00	—	76	"
Guinea pig	300	5.84	3.4	102	<1.4
"	290	4.67	2.9	95	<1.3

* Calculated from data obtained by the method of Burns and Evans(7).

ing the specific activity of tissue L-ascorbic acid 24 hours after administration of the labeled compound. The % conversion was calculated by multiplying the specific activity of the tissue L-ascorbic acid by the body pool for each species. In experiments with unlabeled 2-keto-L-gulonic acid and its methyl ester, the 0-24 urine was collected in 10 ml of 10% oxalic acid as a preservative. L-ascorbic acid was titrated with indophenol dye.

Results. It was found that less than 1.2% of the C¹⁴ administered to rats, as 2-keto-L-gulonic acid C₆¹⁴, was present in the animal as L-ascorbic acid 24 hours after the dose (Table I). This compares to an average value of 8% obtained in similar experiments in rats with L-gulonolactone-1-C¹⁴(7). The results showing that 2-keto-L-gulonic acid was converted to L-ascorbic acid to a lesser extent than L-gulonolactone suggest that this acid is not an intermediate in the conversion of L-gulonolactone to L-ascorbic acid. The most likely intermediates involved in this *in vivo* oxidation are either 2-keto or 3-keto-L-gulonolactone. These compounds, however, have not been isolated since they rapidly enolize to form L-ascorbic acid(1,15,16).

It was also found that less than 1.4% of the C¹⁴ administered to guinea pigs as 2-keto-L-gulonic acid-C₆¹⁴ was present in the animal as L-ascorbic acid 24 hours after the dose (Table I). The lack of appreciable conversion of 2-keto-L-gulonic acid to L-ascorbic acid in guinea pigs is in agreement with results showing that this compound has no vit. C activity in guinea pigs(1-4).

[†] The non-labeled 2-keto-L-gulonic acid and its methyl ester were kindly donated by Dr. R. Paster-nack, Chas. Pfizer & Co.

Evidence has been presented for conversion of certain esters of 2-keto-L-gulonic acid to the vitamin. For instance, methyl 2-keto-L-gulonate has vit. C activity in guinea pigs and in addition increases urinary excretion of L-ascorbic acid when administered to man and dog(1-4). In the present study, data were obtained in the rat for transformation of methyl 2-keto-L-gulonate to L-ascorbic acid. This was shown as follows: 4 rats which excreted daily $0.9 \pm .02$ mg of L-ascorbic acid, received by intraperitoneal injection 90 mg of 2-keto-L-gulonic acid and a week later 90 mg of methyl 2-keto-L-gulonate. No appreciable increase in the daily urinary L-ascorbic acid excretion was observed following administration of the acid ($.5 \pm .1$ mg) but following administration of the ester, the urinary excretion of L-ascorbic acid increased to 7.1 ± 1.0 mg/day. Control experiments in which 90 mg of the acid or the ester was added to the preservative, and urine was collected over 24 hours, showed less than 1 mg of each compound converted to L-ascorbic acid. The difference in the *in vivo* conversion of 2-keto-L-gulonic acid and its esters to L-ascorbic acid may be due to differences in the rates of enolization and lactonization of the compounds to L-ascorbic acid(1,5,10,16-18).

The data in Table I show that the major part of the C^{14} administered as 2-keto-L-gulonic acid C_6^{14} was excreted in urine which was found by an isotopic dilution method to be (within experimental error $\pm 5\%$) unchanged 2-keto-L-gulonic acid. During 24 hours after injection of the labeled 2-keto-L-gulonic acid, 3% of the C^{14} was recovered in respiratory CO_2 of guinea pigs and 10% in the case of rats.

Summary. Evidence has been obtained employing C^{14} tracer technics which shows no appreciable conversion of 2-keto-L-gulonic acid to L-ascorbic acid by the rat or guinea pig. From these results it is suggested that

the most likely intermediate in the conversion of L-gulonolactone to L-ascorbic acid by the rat is 2-keto- or 3-keto-L-gulonolactone. In addition it was found that the major part of the administered 2-keto-L-gulonic acid was excreted unchanged in the urine.

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Xanthine Oxidase in Pair-Fed Rats on Diets Marginally Deficient in Lysine. (22923)

LUCIEN A. BAVETTA* AND STUART A. NARROD†

National Institute of Dental Research, Bethesda, Md.

A number of studies relating xanthine oxidase activity in the liver of the rat to quality of dietary protein has been carried out (1,2,3). It is well established that in inanition and during periods of restricted protein intake, animals suffer loss of liver protein as well as other tissue protein. This decrease in liver protein, however, does not represent an equivalent decrease in activity of all liver enzymes. Thus a decrease of xanthine oxidase activity (4) and an increase in succinoxidase activity has been reported in rats on reduced food intake for 3 weeks (5).

Procedure. We attempted to resolve factors associated with inanition by using paired feeding technic with weanling rats. All experimental groups were pair-fed to the group receiving the lowest amount of amino acid (0.25% lysine). The caloric intake was identical in all groups. Weanling rats were used to give a better insight on the influence of lysine on xanthine oxidase synthesis during feeding of an isocaloric diet. It was hoped that the initially low content of xanthine oxidase in weanling rats (6) would demonstrate larger differences in enzyme content with growth, rather than enzyme depletion demonstrated in adult rats (7). Composition of basal diet is given in Table I. The experimental groups were arranged so that rats in group I had 0.25%; group II, 0.5% and group III, 1.00% L-lysine supplementation. The diets were made isonitrogenous by addition of glycine. Each group contained 9 male weanling rats of Sprague-Dawley strain from the colony of the National Institutes of Health. Our previous experiments had established the adequacy of growth on both 1.0% and 0.5% L-lysine, but the animals on the

TABLE I. Composition of Basal Diet.

	g
Zein	24
Salt H.M.W.	4
Cerelose	51.8
Choline chloride	.2
Vitamin mix 80*	1
" " 90†	5
" " 60‡	8
Amino acid supplement§	6

* Ascorbic acid 10 g, sucrose 490 g.

† Thiamin HCl, 20 mg; riboflavin, 20 mg; pyridoxine, 20 mg; calcium pantothenate, 50 mg; niacin, 50 mg; biotin, 2 mg; folic acid, 5 mg; inositol, 400 mg; p-aminobenzoic acid, 200 mg; vit. B₁₂, .15 mg; menadione, 5 mg; liver powder, 2.0 g; to 50 g with dextrin.

‡ Vit. A, 5,500 i.u.; vit. D, 1,100 i.u.; alpha tocopherol, 200 mg; cottonseed oil to 80.00 cc.

§ DL-tryptophan, .3 g; DL-methionine, .4 g; DL-phenylalanine, .5 g; L-histidine HCl, .4 g; DL-threonine, .6 g; DL-valine, 1.6 g; DL-isoleucine, 1.0 g; L-arginine, .2 g.

0.25% L-lysine barely maintained an increase of 6 g per week when fed *ad libitum*. The immediate and prolonged growth response obtained on these rations when adequately supplemented with L-lysine indicates that the deficiency is of an uncomplicated nature. Animals were placed in metal cages with raised screen bottoms and had unlimited access to distilled water. After 42 days they were lightly anesthetized, decapitated and the livers quickly removed and placed on cracked ice for several minutes. The livers were blotted free of moisture and a weighed portion of each was homogenized in 2 volumes of ice-cold M sodium potassium phosphate buffer (pH 7.3). Liver xanthine oxidase was determined according to the method of Axelrod and Elvehjem (8). Each pair of Warburg flasks contained 2 ml of a liver homogenate, total volume of 3 ml. The endogenous respiration was partially exhausted by a preliminary 10 minute incubation before 0.15 ml of 0.05 M xanthine substrate was tipped into the flask. The protein content of liver homogenate was determined by the method of Lowry *et al.* (9).

* Visiting Scientist from Dept. of Biochemistry, University of S. California, Los Angeles.

† Research Associate, Am. Dental Assn. at Natl. Inst. of Dental Research, N.I.H., P.H.S., U. S. Dept. of Health, Bethesda, Md.

TABLE II. Relationship of Liver Xanthine Oxidase Activity to Lysine Deficiency in the Rat.

Group	L-lysine supple- menta- tion, %	Activity/g		% protein of total wet liver sample
		Liver protein μl O ₂ /hr	Wet liver	
I	.25	87	11	12.7
II	.5	994	170	17.1
III	1.0	689	170	24.6

Results are summarized in Table II. The findings indicate less xanthine oxidase per unit weight of liver in group I as compared to groups II and III. When expressed as activity per unit of liver protein, there is an apparent increase in activity of group II over group III. However, the activity, as per unit of protein, of group I still demonstrates a most marked decrease compared to activity of the other 2 groups. Reduction of observed activity at the 0.25% L-lysine level, even when expressed in terms of unit protein, would indicate that a preferential synthesis of at least some protein components occurred at the expense of xanthine oxidase, unlike those animals on a 0.5% L-lysine diet where reduced liver protein synthesis did not include this enzyme. In these experiments reduced xanthine oxidase appears to be directly related to amount of lysine supplementation and was uncomplicated by factors of inanition.

In general, the results correlate well with previous studies concerning effects of amino acid deficiencies on liver enzyme activities (10,11,12). The increased xanthine oxidase activity per unit protein at the 0.50% L-lysine level, however, does not indicate the

same degree of lability of this enzyme as during the "sub-acute food restriction" observed by Wainio *et al.* (13).

Summary. Weanling rats maintained on diets marginally deficient in lysine appear to exhibit a defect in the ability to synthesize xanthine oxidase. These findings give further support to the importance of dietary lysine for xanthine oxidase activity.

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Interconversion of Δ^1 -Cortisone and Δ^1 -Hydrocortisone in Man.* (22924)

DAVID F. JOHNSON,[†] ERICH HEFTMANN[‡] AND JOSEPH J. BUNIM

Nat. Inst. of Arthritis and Metabolic Diseases, N. I. H., Bethesda, Md.

The interconversion of cortisone (E) and hydrocortisone (F) in man is well established(1,2,3). The dramatic therapeutic effect of their Δ^1 -analogs, first described by Bunim *et al.*(4) has stimulated investigations into the study of the *in vivo* metabolism of these compounds. Recent preliminary reports have indicated that Δ^1 -cortisone (Δ^1 E) is converted to Δ^1 -hydrocortisone (Δ^1 F)(5,6). The present study extends the applicability of the method of determining individual adrenocortical hormones, developed in this laboratory(7,8), to the urinary corticosteroid excretion in patients treated with Δ^1 E and Δ^1 F.

Methods. Details of urine extraction and partitioning of extracts are given elsewhere (8). In principle, the 24-hr urine sample is adjusted to pH 1, extracted with chloroform, and the concentrated extract partitioned on a water-impregnated silicic acid column, using gradient elution with petroleum ether containing increasing amounts of dichloromethane. The dried eluate fractions are dissolved in 95% ethanol, an aliquot is analyzed by ultraviolet (u.v.) absorption at 240 $m\mu$ and another aliquot by Blue Tetrazolium (B. T.) reduction(9). Excretion patterns are obtained by plotting tube numbers against optical density for both assays. The identity of isolated chromatographic bands is confirmed by paper chromatography.

Results. A man with rheumatoid arthritis

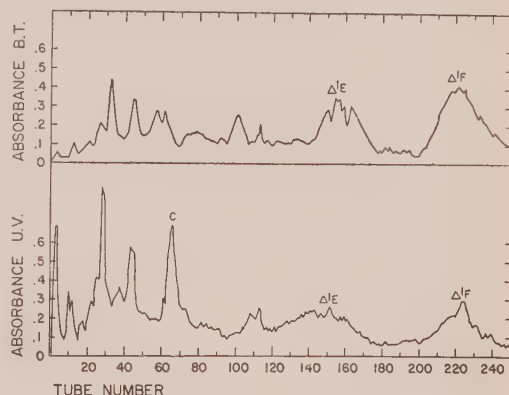


FIG. 1. Corticosteroid excretion pattern after administration of Δ^1 -hydrocortisone.

was given 15 mg of Δ^1 F/day for 4 days. The 24-hr urine extracts of this period were pooled and gave the pattern in Fig. 1. Paper chromatography of Fraction 130-180 in the toluene-propylene glycol system of Zaffaroni(10) for 96 hrs gave a spot with a mobility of 7.0-10.1 cm compared with mobilities of 9.3-12.0 cm for E and 7.0-10.0 cm for Δ^1 E. In the toluene-ethyl acetate-methanol-water system of Bush(11), this fraction gave one spot with an Rf of 0.48 compared with 0.48 for Δ^1 E and 0.55 for E. F and Δ^1 F could not be separated in 96 hrs in the Zaffaroni system. However, in the Bush system Fraction 200-250 gave a spot with an Rf value of 0.21 as compared to 0.23 for Δ^1 F and 0.31 for F. Both fractions exhibited an absorption maximum near 240 $m\mu$ and reduced both Tollens and B. T. reagents. After spraying the chromatograms with 10% aqueous NaOH and drying them for 1 hr at 60°C neither fraction gave the primrose fluorescence shown by E and F (11). The qualitative tests and chromatographic comparisons with known compounds indicate the identity of Fraction 130-180 with Δ^1 E and of Fraction 200-250 with Δ^1 F.

Quantitative estimations were made by referring absorbances to standard curves for the u.v. and B.T. assays of pure Δ^1 E and Δ^1 F. The amount of Δ^1 E was 2.0 mg by u.v.

* The authors gratefully acknowledge advice and criticism of Dr. Erich Mosettig and a supply of Δ^1 E (Prednisone) and Δ^1 F (Prednisolone) by Schering Corp., Bloomfield, N. J.

While this manuscript was in preparation, 2 papers (12,13) on this subject have appeared which confirm our observations.

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[‡] Presented at Internat. Cong. of Clin. Chem., New York City, Sept. 14, 1956.

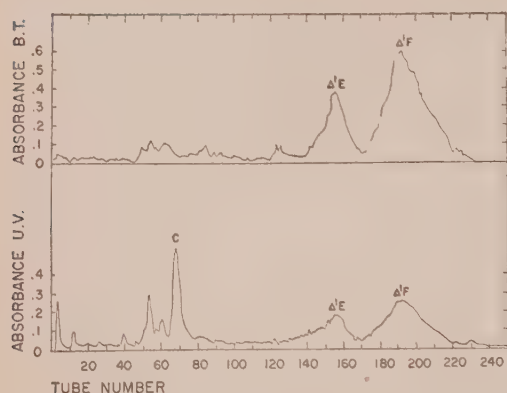


FIG. 2. Corticosteroid excretion pattern after administration of Δ^1 -cortisone.

assay and 1.1 mg by B.T. For Δ^1 F the amount was 1.6 mg by u.v. assay and 1.3 mg by B.T.

Fig. 2 represents the corticosteroid excretion pattern of a woman with multiple myeloma who received 50 mg of Δ^1 E. Paper chromatography of Fraction 135-170 in the Bush system gave one spot with an Rf of 0.48 compared with 0.48 for Δ^1 E and 0.55 for E. In the same system, Fraction 171-220 gave one spot with an Rf of 0.25 compared with 0.26 for Δ^1 F and 0.34 for F. Qualitative tests were carried out as described above. The amount of Δ^1 E was 0.9 mg by u.v. assay and 0.6 mg by B.T. The amount of Δ^1 F was 1.5 mg and 1.6 mg by u.v. and B.T. assays respectively. The high value in the u.v. assays of Δ^1 E indicates in both cases interference with the analysis by this method.

The peak labeled C in both figures had a maximum ultraviolet absorption at 273 m μ and was identified as caffeine(8).

Discussion. The high therapeutic and glucocorticoid activity of Δ^1 E led Gray *et al.*(5) to postulate that this effect might be due to *in vivo* conversion of Δ^1 E to E. However, they obtained paper chromatographic evidence of the excretion of unchanged Δ^1 E and Δ^1 F, but not E or F. Subsequent work by Vermeulen(6) confirmed the conversion of Δ^1 E to Δ^1 F. The results of our experiments

furthermore indicate that Δ^1 E and Δ^1 F are interconvertible in the body and that the order of magnitude of excreted free Δ^1 E and Δ^1 F is similar after the administration of either compound. Analogous results have been reported in metabolic studies of the parent compounds E and F(1,2,3). Our results as well as others(5,6) indicate no excretion of E and F as a result of reduction of Δ^1 E and Δ^1 F. This does not exclude the possibility that E and F are formed. If formed, they may be further reduced to the tetrahydro derivatives which are excreted in conjugated form. Glucuronidates were not hydrolyzed in this study.

Summary. A method of determining individual adrenocortical hormones by partition chromatography on columns is applied to the study of urinary corticosteroid excretion in patients treated with the synthetic steroids Δ^1 -cortisone and Δ^1 -hydrocortisone. Chromatographic evidence indicates *in vivo* interconversion of the two compounds in man.

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Suppression of Prednisone-Induced Gastric Hypersecretion by an Anticholinergic Drug.*† (22925)

J. V. CARBONE,‡ D. LIEBOWITZ, AND P. H. FORSHAM.

(With technical assistance of Valda Crevling) (Introduced by T. L. Althausen)

*Department of Medicine, and Metabolic Unit for Research in Arthritis and Allied Diseases,
University of California School of Medicine, San Francisco.*

While the stimulatory effects of corticoids on gastric secretion have been well established, the exact mechanism remains unknown (1). The complications of acute perforation and hemorrhage following the activation of peptic ulcers during administration of corticoids (1-4) in many instances have precluded the therapeutic use of these drugs. This study was undertaken to determine whether the anticholinergic drug, oxyphenonium bromide,§ would affect the gastric hypersecretion induced by the synthetic corticoid, prednisone.

Methods. Ten normal male medical students, aged 22 to 30 years, were selected as subjects because they showed a definite rise in gastric acidity when given prednisone orally. The experiment was divided as follows: 1) a 7-day period during which 8 subjects received 40 mg of prednisone daily in 4 equally divided oral doses, 2) a 7-day period during which these 8 subjects received 40 mg of prednisone and 40 mg of oxyphenonium bromide daily in 4 equally divided oral doses, 2) a 7-day period during which 5 of the 10 subjects received 40 mg of prednisone and 20 mg of oxyphenonium bromide daily in 4 equally divided oral doses, 3) a 7-day period during which 5 of these subjects received 20 mg of oxyphenonium bromide and 5 received 40 mg of oxyphenonium bromide in 4 equally divided oral doses. The study periods were separated

by recovery periods of 2 to 4 weeks. The gastric contents of the fasting subjects were aspirated during 4 consecutive 15-minute intervals before and after administration of the drugs. The volume and the free hydrochloric acid concentration were determined, and the total content of hydrochloric acid was calculated. Twenty-four-hour urine output was collected for uropepsin determinations (5) prior to and on the last day of each period.

Results. As shown in Fig. 1, oxyphenonium bromide significantly suppressed hydrochloric acid secretion when given alone and when given in conjunction with prednisone ($P = .001$). In both cases the suppression was quantitatively similar. The drug was equally effective at the 20 and 40 mg dose level.

Uropepsin excretion rose significantly in 8 subjects following the administration of 40 mg of prednisone ($P = .01$). A significant suppression ($P = .06$) occurred in all subjects when oxyphenonium bromide was administered simultaneously (Fig. 2). The two instances in which the uropepsin level remained above control levels (columns 4 and 6) occurred in individuals whose uropepsin excretion was 19,000 and 24,000 units, respectively, during administration of prednisone.

Discussion. The unresponsiveness of ACTH-induced hypersecretion to anticholinergic drugs has been reported (6). The successful clinical use of such agents in preventing peptic ulceration in patients treated with large doses of prednisone was described recently by Dubois (7). According to our results, the conjunctive use of the anticholinergic drug, oxyphenonium bromide, during administration of prednisone does prevent an increase in gastric hydrochloric acid secretion and uropepsin excretion. The increased gastric secretion induced by corticoids has been

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† The authors wish to thank Professor T. L. Althausen for his continued interest.

‡ Giannini Fellow in Medicine, 1954-1955.

§ Diethyl (2 hydroxyethyl) methylammonium bromide phenylcyclohexaneglycolate (Antrenyl® Ciba).

| Δ1 cortisone (Meticorten®, Schering).

GASTRIC HCl SECRETION

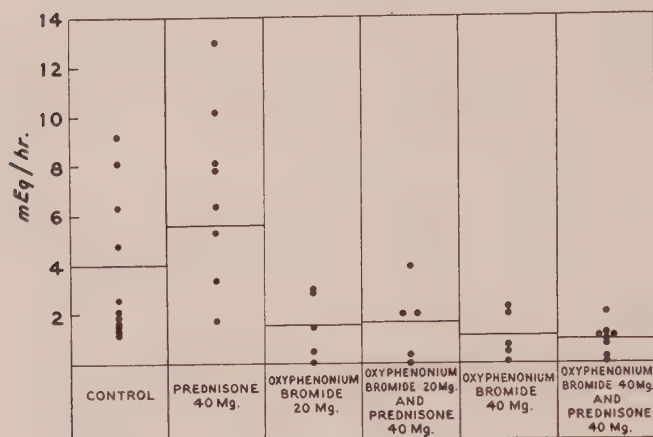


FIG. 1. Effect of oxyphenonium bromide with and without prednisone on gastric secretion.

shown to be independent of vagal innervation (8). Yet, oxyphenonium bromide, which antagonizes acetylcholine, appears to prevent the action of prednisone on gastric secretion.

Apparently, prednisone enhances the effect of acetylcholine either by sensitizing the parietal cell to its action or, more directly, by increasing its production or decreasing its destruction.

UROPEPSIN EXCRETION

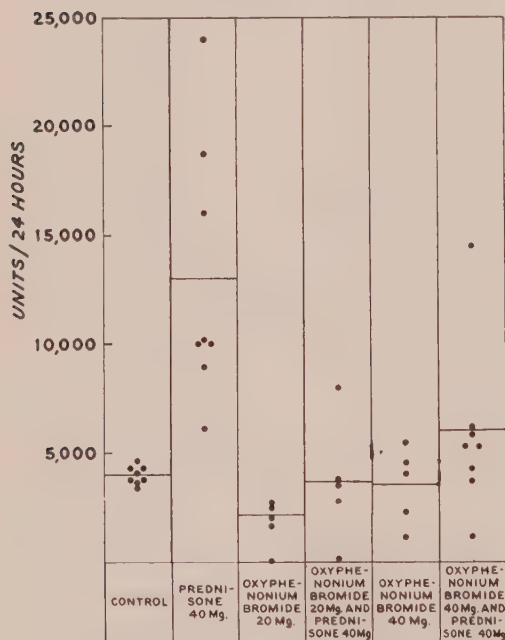


FIG. 2. Effect of oxyphenonium bromide with and without prednisone on uropepsin excretion.

Summary. The effect of an anticholinergic drug was studied in 10 normal males who showed increased gastric secretory activity during administration of prednisone. It was found that concurrent administration of oxyphenonium bromide (Antrenyl®) suppressed the increase in gastric hydrochloric acid secretion and reduced uropepsin excretion.

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Effect of Cortisone on Mouse Resistance to Intravenous Toxicity of Influenza Virus.*† (22926)

NEWTON KHOBYARIAN† AND DUARD L. WALKER. (Introduced by C. V. Seastone)

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, and Naval Medical Research Institute, Bethesda, Md.

While cortisone and ACTH have, in general, been found to depress host resistance to bacterial and viral infections(1) no uniform pattern has been found for effects of these hormones upon host resistance to toxins of bacteria, rickettsiae, and viruses. Although effects on reactions to bacterial toxins have been studied by several investigators in some detail(1), very few studies have been reported involving the so-called toxic reactions produced by rickettsial and viral agents. Jackson and Smadel(2) found that cortisone and ACTH did not alter the reactions of mice to several rickettsial toxins and Kass and co-workers(3) found no effect of ACTH upon intravenous toxicity in mice and rats of influenza A virus or agents of murine and epidemic typhus. There has been one report(4) of increased reactivity of cortisone treated mice to the intracerebral toxicity of influenza B virus.

In the course of studies on tissue damaging effects of influenza virus and factors which alter these effects it was found that appropriate treatment of mice with cortisone increased their susceptibility to the acute lethal effects of large quantities of intravenously injected virus. In view of the paucity of information on the effect of cortisone on reactions to viral toxins, the following studies were carried out.

Materials and methods. The PR8 strain of influenza A, Lee strain of influenza B, and Victorian strain of Newcastle disease virus (NDV) were cultivated in the allantoic sac of embryonated eggs. Allantoic fluid was stored at -70°C until it was shown to be free

of bacterial contamination by culture in thioglycollate and nutrient broth. It was then used undiluted or diluted in physiologic saline buffered to pH 7.2 with phosphate. Measurement of the number of agglutinating doses (A.D.) in these preparations was by a pattern technic with a final concentration of 0.5% chicken erythrocytes. Cortisone acetate (Cortone, Merck) was injected subcutaneously in 0.1-0.2 ml volumes. Physiologic saline containing 1.5% benzyl alcohol and buffered to pH 7.2 with phosphate was used in place of cortisone for control injections in most experiments. In some experiments a solution incorporating the suspending agents used in the Merck product was employed. There was no evidence of a difference in the effects of these two control solutions. Allantoic fluid from uninoculated eggs was used as a control substitute for infected allantoic fluid. Immune sera were from immunized rabbits and were heated at 56°C for $\frac{1}{2}$ hour before use. Swiss white mice (female, Webster strain) weighing 10-13 g and 3-4 weeks old were used. Virus suspensions were injected into the tail vein in 1.0 ml volumes. Only deaths occurring within 72 hours after viral injection were considered as due to toxic effects of the virus.

Results. Groups of mice were given 2.5

TABLE I. Effect of Cortisone on Toxicity of IV Injected PR8 and Lee Influenza Viruses and NDV in Mice.

Preliminary treatment	Challenge (i.v.)	No. dead* /No. inj.
Control fluid	PR8 influenza, 500 A.D.	3/20
Cortisone†	<i>Idem</i>	19/20
Control fluid	Lee influenza, 2560 A.D.	4/20
Cortisone	<i>Idem</i>	19/20
Control fluid	NDV, 2560 A.D.	4/20
Cortisone	<i>Idem</i>	17/20
"	Normal allantoic fluid	0/20
"	56°C heated PR8, 2500 A.D.	0/20

* Observation period: 72 hr.

† Mice received 2.5 mg subcut. 24 hr and 3 hr before challenge.

* Opinions expressed in this article are those of the authors and do not necessarily reflect views of the Navy Department.

† The authors are indebted to W. I. Capps, HMC, USN and E. C. Sutor, HM2, USNR for their able technical assistance in this work.

‡ Present address: Indiana University Medical Center, Indianapolis.

mg of cortisone 24 hours and again 3 hours before intravenous injection of virus suspensions. Control groups of mice were given an equal volume of 1.5% benzyl alcohol in saline on the same schedule. It was found (Table I) that although the doses of viruses selected for intravenous challenge produced only a few deaths in each control group, many more mice died of the acute effects of intravenous PR8 influenza A, Lee influenza B, and Newcastle disease virus in those groups treated with cortisone. PR8 influenza virus previously heated at 56°C for ½ hour had no apparent toxicity even when given in very large quantities to cortisone treated mice. Comparing the effects in control and cortisone treated groups, P for chi-square was less than 0.001 for each of the active viruses tested indicating that the results were highly significant.

To determine whether cortisone treated and control mice differed to this degree in their susceptibility to a range of doses of virus, control and cortisone treated mice in groups of 20 were injected intravenously with stepwise dilutions of a pool of PR8 infected allantoic fluid. Dilutions were made in 0.15 log₁₀ steps. The results are presented in Fig. 1 and it can be seen that for both cortisone

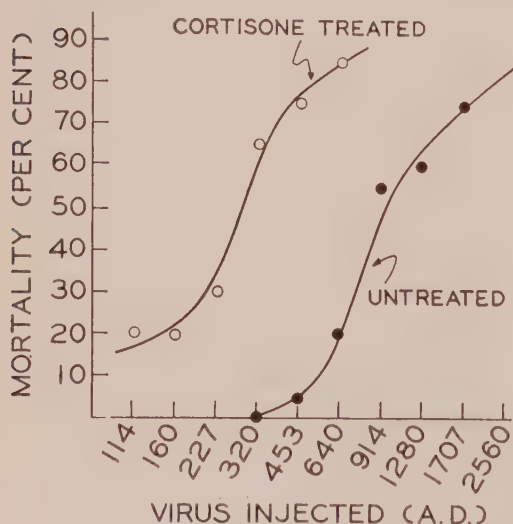


FIG. 1. Effect of cortisone in reducing resistance of mice to a range of doses of intravenous PR8 influenza A virus. Mortality is that occurring within 72 hr after virus injection into groups of 20 mice.

TABLE II. Effect of Immune Serum on IV Toxicity of PR8 Influenza Virus in Cortisone Treated Mice.

1st inj.	Time interval	2nd inj.	No. dead /No. inj.
PR8 I.S.* + PR8 virus†	—	None	0/20
Normal serum	1 hr	PR8 virus	19/20
PR8 immune serum	"	<i>Idem</i>	1/20
Lee " "	"	"	18/20
PR8 virus	15 min.	Saline	18/20
<i>Idem</i>	"	PR8 immune serum	7/20
"	"	Normal serum	16/20
"	3 hr	PR8 immune serum	19/20
"	"	Normal serum	17/20

* Rabbit PR8-immune serum.

† 640 A.D.

treated and control animals the points, representing the percentage of deaths in each experimental group, fall into curves roughly sigmoid in shape and that these are approximately parallel throughout their courses.

It was important to determine whether the lethal effect of intravenous influenza virus for cortisone treated mice could be prevented by addition of specific immune serum to the virus inoculum or by passive immunization of mice before or after virus injection. An experiment designed to test this is outlined in Table II. A mixture of 640 A. D. of PR8 virus and PR8 antiserum in 1:20 final dilution was without lethal effect when injected into cortisone treated mice. PR8 antiserum (0.2 ml, undiluted) given by intravenous injection one hour before PR8 virus had a marked protective effect as compared to normal serum or Lee antiserum. When specific antiserum was injected intravenously only 15 minutes after an injection of virus the number of resulting deaths was reduced somewhat but when antiserum administration was delayed for 3 hours it had no significant effect on the outcome.

Stained smears and cultures in thioglycolate broth and on blood agar of blood and tissues from cortisone treated mice gave no evidence that bacterial infections activated by cortisone treatment played any part in these results.

To determine the influence of quantity of

TABLE III. Effect of Dose Size and Time of Cortisone Treatment on Resistance of Mice to IV Toxicity of PR8 Influenza Virus.

Cortisone treatment, mg (s.e.)				No. dead/No. inj.
Hr before challenge*				
72	48	24	3	
		C.f.†	C.f.	1/20
		.5	.5	7/20
		1.0	1.0	10/20
		2.0	2.0	16/20
		2.5	2.5	18/20
.5	.5	.5	.5	17/20
5.0				9/20
	5.0			17/20
		5.0		16/20
			5.0	3/20

* 500 A.D. of PR8 influenza i.v.

† Control fluid (0.2 cc).

cortisone and time of cortisone treatment relative to virus injection, groups of mice were treated with cortisone by several schedules and then injected with 500 A.D. of PR8 influenza virus. The most important of these groups are presented in Table III. Control mice for each of the cortisone schedules were treated with cortisone but injected with normal allantoic fluid. No deaths occurred in cortisone treated controls and details of these controls are, therefore, not included in the table. It was found that when cortisone was given in two injections, one 24 hours and one 3 hours before virus injection, susceptibility was increased as cortisone dosage was increased from 0.5 mg to 2.5 mg per injection. Larger doses had little further effect on susceptibility to viral toxicity and resulted in some deaths apparently due to the cortisone itself. A slight increase in susceptibility to viral toxicity was demonstrable when a third dose of 2.5 mg of cortisone was given 24 hours after virus injection. Although a total of 2.0 mg of cortisone given in two 1.0 mg doses beginning 24 hours before virus injection resulted in only 10 deaths in a group of 20 mice, the number of mice succumbing was increased to 17 when 2.0 mg was given in 0.5 mg doses beginning 72 hours before challenge. The importance of a sufficient interval between the initiation of cortisone treatment and injection of the virus challenge was further indicated by the finding that as much as 5 mg of cortisone given 3 hours before challenge had little effect on susceptibility while

5 mg given 24 or 48 hours before challenge markedly increased susceptibility. The influence of a single 5 mg injection of cortisone had apparently begun to wane when challenge was deferred for 72 hours.

It was of interest to see if lesions produced in cortisone treated mice were different in location and characteristics from those in untreated mice. During the course of several experiments in which graded doses of PR8 influenza were employed mice from cortisone treated and control groups were killed at 6-12 hour intervals and examined for gross and microscopic pathology. Gross and microscopic lesions appeared in cortisone treated mice with lower doses of virus; but their time of appearance (6-12 hours after challenge), location and characteristics were essentially similar to those seen in untreated mice given at least four-fold greater quantities of virus. The lesions produced in mice by intravenous injection of influenza virus have been described previously(5) and details need not be repeated here. In cortisone treated mice, as in untreated controls, the major damage was found in the liver.

Discussion. The experiments described show that cortisone lowers resistance in mice to the intravenous toxicity of influenza viruses and NDV providing cortisone treatment is begun 24 hours or more before challenge. How this is brought about is not yet apparent. Since in these experiments only deaths occurring within 72 hours after challenge were considered as due to viral toxicity and since passive immunization of cortisone treated mice 3 hours after viral injection had no effect on the outcome, it appears unlikely that suppression of antibody production by cortisone was of any importance as a cause of the increased susceptibility. The similarity of location and characteristics of lesions produced in cortisone treated and untreated mice suggest that it was not due to additional tissues becoming susceptible to viral damage. The appearance of histologic evidence of tissue damage with smaller doses of virus in cortisone treated mice as compared to controls suggests that affected cells may be damaged by smaller quantities of virus, or that

under the influence of cortisone the cells may support some degree of multiplication to reach levels of virus which result in cellular destruction. Work is being continued to explore these possibilities.

Summary. 1. Treatment of mice with cortisone significantly reduced their resistance to the intravenous toxicity of PR8 influenza A, Lee influenza B, and Newcastle disease viruses. 2. Deaths of cortisone treated mice were prevented by mixing specific immune serum with the viral inoculum or by passive immunization of mice prior to challenge. Immune serum injected intravenously 15 minutes after challenge gave some protection but was without effect when injected 3 hours after challenge. 3. For full effect, doses of

2.5 mg of cortisone had to be used when treatment was begun 24 hours before challenge. Smaller doses were effective if begun earlier. Doses as large as 5.0 mg had no significant effect if given only 3 hours before challenge.

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Progestational Activity of 19-norprogesterone and 19-norethisterone in the Rhesus Monkey. (22927)

WILLIAM W. TULLNER AND ROY HERTZ

Natl. Cancer Inst. N.I.H., U. S. Public Health Service, Bethesda, Md.

The synthesis of 19-norprogesterone and 19-norethisterone has been previously reported(1,2). These 19-nor steroids have been shown to possess high progestational potency(3,4). 19-norprogesterone was found to be 4 to 8 times as active as progesterone when administered parenterally(3). The degree of endometrial proliferation in the rabbit uterus was used as the criterion of progestational activity. Oral administration of norethisterone (17 α -ethinyl-19-nortestosterone) gave a five-fold increase in activity over that of orally administered ethisterone (17 α -ethinyltestosterone) in the rabbit assay(4).

This report concerns further observations on these compounds with respect to their relative ability to produce progestational effects in the ovariectomized rhesus monkey.

Materials and methods. Bilaterally ovariectomized rhesus monkeys (*Macacus rhesus*) weighing 3-4 kg were given daily subcutaneous injections of 10 μ g estradiol-17 β in 0.25 cc sesame oil for 20 days. Beginning on the twenty-first day, daily saline vaginal

lavage was used to determine the onset of withdrawal bleeding. This procedure was continued until withdrawal bleeding was completed. The interval between cessation of estrogen administration to the beginning of estrogen-withdrawal bleeding was checked at least twice in each animal before determining the effectiveness of the test compounds in preventing estrogen-withdrawal bleeding. After pretreatment with estrogen as previously indicated, daily administration of test compounds was begun on the twenty-first day. Ethisterone and norethisterone were given orally in tablet form (except as noted in Table I). Solutions of 19-norprogesterone and progesterone were administered subcutaneously in sesame oil. The onset and completion of withdrawal bleeding were determined as noted above.

Results. In Table I, 19-norprogesterone and progesterone have been compared with respect to the characteristic inhibition of estrogen-withdrawal bleeding by progesterone. There was a delay in withdrawal bleeding of

TABLE I. Effect of 19-norprogesterone and Progesterone on Estrogen-Withdrawal Bleeding in the Ovariectomized Rhesus Monkey

Treatment	Daily dose (mg)*	Delay of bleeding (days)	Normal delay of estrogen-withdrawal bleeding (days)
19-norprogesterone	.125	16†	4, 5, 6
	"	31†	5, 8, 7
	"	31†	3, 5, 5
	.0625	11	5, 5, 7, 6, 5
	"	12	5, 6, 7, 5
	.0312	8	8, 8
	"	7	6, 7
	"	8	8, 6
Progesterone	1.0	42	7, 7, 8
	"	13	7, 7, 6
	"	30	7, 6, 7

* Administered in 0.2 cc sesame oil solution daily subcut.

† Treatment discontinued. Menstrual bleeding occurred 3-4 days later.

13 to 42 days in 3 monkeys receiving daily subcutaneous injections of 1 mg progesterone. A substantial delay in the onset of bleeding was produced by daily injection of 0.125 mg 19-norprogesterone in each of 3 animals. A smaller dose of the latter compound (0.0625 mg) had a marginal effect on the suppression of bleeding whereas one-half this dose (0.031 mg) is subthreshold and menstrual bleeding in this group is within the normal range of estrogen-withdrawal bleeding time in the monkey treated with estrogen alone.

The data for the inhibition of estrogen-withdrawal bleeding by progesterone are in accord with the earlier studies of Hisaw and Greep(5). These authors reported a 9- to 44-day delay of withdrawal bleeding when 1.0 mg progesterone was given subcutaneously daily.

As previously noted, results obtained with the Clauberg rabbit method had indicated that 19-norprogesterone was 4 to 8 times more potent than progesterone. The work of Mardones *et al.*(6) has shown 19-norprogesterone to be more potent than progesterone with respect to antiluteinizing, antihypertrophic and antifibromatogenic activity in the guinea pig. Results of the present study indicate that 19-norprogesterone is 8 to 16 times more active than progesterone in delay-

ing the onset of estrogen-withdrawal bleeding in the monkey.

Table II presents a summary of experiments in which 19-norethisterone and ethisterone are compared for ability to inhibit uterine bleeding induced by the withdrawal of estrogen.

When these compounds were administered orally, a daily dose of 5 mg norethisterone prevented uterine bleeding until treatment was discontinued. After 15 days treatment with norethisterone (5 mg daily), the uterus of one monkey was biopsied. Histological examination showed a progestational type of endometrium. After 9 days of additional treatment the endometrium exhibited an excellent decidual response. As little as 2.5 mg norethisterone daily produced a definite delay of uterine bleeding in 2 of 3 animals. A daily dose of 1.25 mg was ineffective in maintaining the endometrium. Ethisterone, on the other hand, failed to effect a signifi-

TABLE II. Effect of 19-norethisterone and Ethisterone on Estrogen-Withdrawal Bleeding in the Ovariectomized Rhesus Monkey.

Treatment	Daily dose (mg)*	Post-treatment delay of bleeding (days)	Normal delay of estrogen-withdrawal bleeding (days)
Norethisterone	5	16†	8, 8
	5	20†	5, 7, 8, 7
	5	20†	5, 5, 7, 5
	5	20†	5, 5, 9, 5
	2.5	8	5, 5, 6
	"	14	5, 6, 7, 7
	"	17	5, 5, 6, 8
	1.25	11	8, 8
	"	9	7, 7
	"	6	5, 7
Ethisterone	5†	5	7, 7
	5†	9	5, 8, 7
	5†	8	5, 5, 7, 6
	25	4	3, 5, 5
	"	9	5, 5, 7, 6
	"	7	5, 6, 7, 5
	100	7	5, 5, 9, 5
	"	7	4, 8
	200	7	5, 7, 8, 7
	"	4	5, 5, 7, 5
	"	6	5, 5, 9, 5

* Administered orally by tablet (except as noted).

† Treatment discontinued at this time.

‡ Administered by stomach tube in 5 cc propylene glycol.

cant delay in estrogen-withdrawal bleeding when given in doses ranging from 5 to 200 mg daily. The failure of ethisterone to produce a delay in the onset of bleeding in the rhesus monkey is noteworthy in view of the demonstration of progestational activity in women of an oral dose of ethisterone 6 to 8 times that of the effective intramuscular dose of progesterone(7,8). In addition, ethisterone has been shown to have marked progestational activity in the rabbit(4,9).

Hertz *et al.* have reported that norethisterone is about 4 times more active than ethisterone in the production of progestational changes of the endometrium in women(10). From the data presented here it appears that norethisterone given orally in the monkey has approximately 40% of the activity of progesterone given by the subcutaneous route.

Summary. Two 19-nor steroids have been tested for progestational activity, as measured by inhibition of uterine bleeding following estrogen-withdrawal in the rhesus monkey. 19-norprogesterone was found to be 8 to 16 times as active as progesterone when administered parenterally. Orally adminis-

tered 17 α -ethinyl-19-nortestosterone was active at 2.5 mg daily whereas 17 α -ethinyl testosterone was inactive in a range of dosages from 5 mg to 200 mg.

It is a pleasure to acknowledge the fine technical assistance of Mr. D. Barber and Mr. J. Brice. We also wish to thank Chemical Specialties Co., for the steroids used.

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Elevated Chylomicron Index in Multiple Sclerosis and Its Response to Digestive Enzyme Therapy.* (22928)

RALPH G. SKILLEN, CLINTON H. THIENES AND ALAN J. FRIEDMAN.

Institute of Medical Research, Huntington Memorial Hospital, Pasadena, and University of S. California, Los Angeles.

The possibility of an altered lipid metabolism in multiple sclerosis has been suggested by the observations of several groups of investigators. Swank(1) found that a low-fat diet appears to reduce frequency and severity of exacerbations of this disease. Roboz, *et al.* (2) have shown by paper electrophoresis a tendency for greater variability in serum lipo-

protein patterns of a series of multiple sclerosis patients than in those of normal individuals; this increased variability appears to be associated with a trend toward increased alpha-1 lipoprotein and corresponding decrease in the lipids of the "gamma" region. Aird, *et al.* (3) produced preliminary evidence which indicates that the relative concentration of the Sf(12-20) serum lipoproteins is elevated in those cases of multiple sclerosis graded "rapidly progressive." Zinn and Grif-fith(4) have demonstrated that in two conditions in which there exists an altered lipid

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metabolism, atherosclerosis and diabetes mellitus, the chylomicron index is elevated. The chylomicron index is the percentage of serum fat particles which appear by dark-field microscopy to be approximately 0.5μ or greater in diameter.

This report presents evidence of an elevated chylomicron index associated with multiple sclerosis and a means whereby this symptom may be reversed toward or into the normal range.

Methods. Blood samples were obtained from subjects at approximately 9:00 a. m. after a fast period starting after lunch the day before. From noon until 10:00 p. m. of day of fast, the subjects were strictly limited in their fat consumption. They were allowed only plain jello, black coffee, tea, citrus and pineapple juice, and water. From 10:00 p. m. until the sample was taken the next morning, the subject was allowed nothing to eat or drink and was not permitted to smoke. Blood was drawn from puncture wound in the side of finger into several capillary tubes of approximately 1 mm bore. These tubes were fire-sealed at the clean end, and after clot had formed and retracted, they were centrifuged at 1,000 r.p.m. for 10 minutes to separate serum from clot. The serum was then transferred to a clean glass slide, and a cover slip placed on the serum. Dark-field examination of fat particles of the serum was made at 1940x magnification. A net micrometer in the 20x ocular was used to estimate particle size. A count was then made to estimate total number of particles and number of particles of a diameter of 0.5μ or greater which were visible at one level of focus in 2 large squares of the net micrometer. One large square of the net micrometer included an area of $25 \mu^2$. This counting procedure was repeated at 50 locations on the slide so that an area of $2,500 \mu^2$ was examined. In all cases duplicate serum samples were similarly counted. The chylomicron index was then calculated. Initially, following determination of chylomicron index before treatment, patients were paired according to severity of condition into a placebo group and a group which received a commercial preparation con-

taining 150 mg of bile salts and 300 mg of pancreatin in an enteric coating around which is a shell of 250 mg of pepsin bearing a water soluble coating (Entozyme).[†] The placebo tablets simulated the Entozyme tablets in appearance and neither the patient nor the observers knew which preparation was being administered until after all tests of the treated patients had been made. The subjects were required to take 3 tablets 3 times a day after meals. At times varying from 1 to 18 months after patients began taking the tablets, their chylomicron indices were again determined. More recently a third group has been included. They received 300 mg of triple strength pancreatin as an enterically coated tablet[‡] 3 times a day and are being studied in the same manner as those receiving either placebo or Entozyme. This group has varying degrees of severity as the other 2 treatment groups. Age of the patient was not considered in assigning patients to the 3 groups. However, the age ranges for the multiple sclerosis groups and the control group of 12 presumably healthy volunteers were similar in that they all included individuals varying in age from the mid-twenties to the mid-sixties.

Results. Pretreatment chylomicron indices have been obtained on 95 multiple sclerosis patients. Of this number, 28 have not been retested after beginning treatment. Of the remaining 67 patients, 21 received placebo, 34 received Entozyme, and 12 received pancreatin. The results of this study are summarized in Table I. The chylomicron index values of Entozyme treated patients are arranged in this table according to the time interval after initiation of treatment at which they were obtained; therefore, in some cases the subjects included in the 1-2 month group are included also in the 2-3 month and/or in the 5-18 month group. Statistical significance of the differences of the means was determined by the

$$\text{formula CR} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(SE)_1^2 + (SE)_2^2}}. \text{ CR must}$$

[†] A. H. Robins Co., Inc.

[‡] Wilson Laboratories.

TABLE I. Effect of Placebo, Pancreatin, and Entozyme Treatment on Chylomicron Index in Multiple Sclerosis.

	Normal control	M.S. pre-treatment	M.S. placebo treated	—M.S. Entozyme treated—			M.S. pancreatin treated, 1-2 mo
				1-2 mo	3-4 mo	5-18 mo	
No. cases	12	95	21	14	18	16	12
Mean & S.D.	34 ± 4.92	55 ± 5.04	55 ± 5.55	37 ± 6.96	34 ± 5.30	37 ± 7.94	41 ± 11.92
Index range	26-41	37-63	45-62	25-57	26-48	23-52	29-61

have a value of 2 or more for the difference of 2 mean values to be significant. By this standard there is no difference between the values for untreated and placebo-treated multiple sclerosis patients, but a significant difference does occur between these and the values for normal subjects, Entozyme treated patients and pancreatin treated patients, respectively. No correlation was found between chylomicron responses and age of the patient. The normal control group was small in number (12 subjects). However, a much larger number of normal cases has been tested by one of us (RGS) but at a different time and in a different laboratory and are therefore not included in Table I. The values were similar to those of the present 12 cases and to those reported by Zinn and Griffith(4) whose control group had an average chylomicron index of $28\% \pm 6.5\%$.

Of additional interest are 2 patients who, having been on Entozyme for over a year, voluntarily ceased therapy. Just before cessation of therapy, one of these patients had a chylomicron index of 33%; 3 months later, the index had not changed, but at 5 months after cessation of therapy, the chylomicron index was elevated to 57%. The other patient's chylomicron index was found to have increased from 32%, the value immediately before the Entozyme was stopped, to 63% four months later. One month after therapy was reinstituted, both patients again had chylomicron indices within the normal range, 39% and 36% respectively.

Comments. In multiple sclerosis, the chylomicron index is significantly elevated

(55%) and is similar to that found by Zinn and Griffith(4) to be associated with atherosclerosis and diabetes, 55% and 52% respectively. However, whether or not the mechanism producing the elevated chylomicron index in multiple sclerosis is in any way related to that of either atherosclerosis or diabetes is a question which cannot be answered at this time.

The elevated chylomicron index may be considered a symptom of multiple sclerosis. The alleviation of this symptom may or may not reflect a beneficial effect in the patient. In this respect, the evaluation of digestive enzyme therapy in multiple sclerosis must await long term clinical evaluation.

Summary. The chylomicron index is above normal in multiple sclerosis. The oral use of a preparation of pepsin, pancreatin, and bile salts lowers the chylomicron index to normal. Pancreatin alone appears to have a similar effect although, as judged by the standard deviations, less regular than the pepsin, pancreatin and bile salts combination. No response was shown by the group which received placebo.

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Effects of 2-Methylation on Glucocorticoid Activity of Various C-21 Steroids. (22929)

W. E. DULIN, B. J. BOWMAN AND R. O. STAFFORD

Department of Endocrinology, The Upjohn Co., Kalamazoo, Mich.

The wide use of steroids as therapeutic agents has led to attempts to increase or change the biological activity by chemical modification. This has been particularly true of the adrenal glucocorticoids since hydrocortisone was found to be extremely beneficial in the treatment of inflammatory diseases. Introduction of a fluorine atom at the C-9 position of several C-21 steroids produces striking enhancement of biological effectiveness(1,2,3). Unsaturation at the C-1-C-2 bond increases the glucocorticoid activity of hydrocortisone and cortisone(4). More recently it has been shown that the combination of 9 α -fluorine and 1,2-unsaturation in the same molecule further increases the glucocorticoid activity of hydrocortisone(5,6). A methyl group at the C-2 of hydrocortisone or 9 α -fluorohydrocortisone molecule is accompanied by a pronounced increase in biological activity of either compound(7,9). Investigation into the effects of 2-methylation on the activity of other 11-oxygenated C-21 steroids structurally related to hydrocortisone has been carried out. This report presents results of glycogen deposition assays and anti-inflammatory tests on a number of steroids in this series. The work has uncovered some interesting correlations between structure and biological activity.

Methods. Animals. All animals were male rats obtained from the Upjohn colony (Sprague-Dawley ancestry). They were adrenalectomized at body weight of 140-160 g and were maintained on stock diet (Archer dog pellets) and 1% sodium chloride as drinking fluid. **Steroids.** The steroids* were administered as suspension in an aqueous system of 0.5% carboxymethyl-cellulose, 0.4% Tween 80, 1.5% benzyl alcohol, and 0.9% sodium chloride. Suspension was accomplished by grinding crystals and vehicle

together in ground glass tissue homogenizer. **Glycogen deposition test.** Liver glycogens were determined by the anthrone method previously described by Stafford *et al.*(5) on tissues removed 7 hours following a single subcutaneous injection of the compounds. **Anti-inflammatory test.** The test used was previously described by Dulin(6) and depends on the ability of 7 daily injections of a compound to inhibit granuloma formation around subcutaneously implanted cotton pellets. **Experimental design.** The unknown compounds were tested simultaneously with hydrocortisone which was used as a standard and assigned a potency value of 1. When the unknowns were tested at two or more dose levels in parallel with hydrocortisone, potency ratios were determined by the method of Irwin(8). In those assays in which one dose of the unknown was tested against 2 or more doses of standard, the potencies of the unknowns were determined by graphically plotting the hydrocortisone dose-response curve and correlating the dose of standard required to produce a response equal to that produced by the test dose of the unknown. A description of the experimental design of the assays is shown in Table I.

Results. 2-Methylation of hydrocortisone (I) and of 9 α -fluorohydrocortisone (II, III) increased both anti-inflammatory and glycogen deposition activity of the parent steroids. In the case of 11 β , 17 α -dihydroxyprogesterone (IV), although the biological activity of the parent compound was barely demonstrable at the doses used, the 2-methylated compound (V) was 30% of hydrocortisone as an anti-inflammatory agent and 110% on the glycogen test. The 2-methylated derivative (XXIII) of 9 α -fluoro-11 β , 17 α -dihydroxyprogesterone (XXIV) was more potent on the anti-inflammatory test than the parent compound (250% and 67% as active as hydrocortisone respectively). It is further seen

* The compounds used were generously supplied by the Chemistry Department, The Upjohn Co.

TABLE I. Description of Experimental Design of Glycogen Deposition and Anti-Inflammatory Tests Which Are Presented in Table II.

Test	Description of test			Compounds tested by each design*
	No. doses of standard	No. doses of unknown	No. rats per group	
Glycogen deposition	2	2	4-5	I, XIII
	3	3	4-6	II, IV, VI, VIII
	2	3	5-10	IX, XVI, XV, XVIII
	3	8	5-10	III
	2	1	5	V, VII, X, XI
	3	1	6-10	XII, XIV, XVII
Anti-inflammatory	2	2	7-10	I, II, III, V, XV, XVII, XVIII, XXI, XXII
	2	1	7-16	IV, VIII, IX, X, XI, XII, XIII, XIV, XIX, XX, XXIII
	1	1	6	XXIV

* Compound numbers and names are shown in Table II.

that 2-methyl- Δ -6-hydrocortisone (VII) was 270% as effective as hydrocortisone on the glycogen test while its nonmethylated parent (VI) was only 100% of hydrocortisone.

In the case of cortisone (VIII), 2-methylation (IX) reduced the anti-inflammatory and glycogen depositing activities. A similar relationship was found to exist between the 2-methyl analogue of 9 α -fluorocortisone (XVI) and the parent compound. This is shown by the fact that the glycogen activity of the methyl derivative is only 40% of hydrocortisone while activity of fluorocortisone is reported(3) to be much greater than that of hydrocortisone.

The activities of 11-ketoprogesterone (XII) and 11 β -hydroxyprogesterone (XIV) were only very slight by both tests and this was also true of the 2-methyl analogues (XIII and XV). The activities of these compounds were not significantly enhanced by 2-methyl substitution. It could not be determined whether there was an actual decrease in activity induced by 2-methylation since the parent compounds possessed very slight activity.

Compound A or 11-dehydrocorticosterone (X) and its 2-methyl analogue (XI) are related to one another much as cortisone and its analogue. In this case the parent compound was 20% of hydrocortisone on the anti-inflammatory test while the 2-methyl analogue was less than 10% as potent as hydrocortisone. In the glycogen deposition test

compound A was 40% as active as hydrocortisone while the 2-methyl analogue was less than 10%. Introduction of a 2-methyl group had no significant effect on the anti-inflammatory or glycogen deposition activities of corticosterone (XVII, XVIII), 9 α -fluorocorticosterone (XIX, XX), 11 β -hydroxyprogesterone (XIV, XV), or 9 α -fluoro-11 β -hydroxyprogesterone (XXI, XXII).

Discussion. It is apparent from these results (Table II) that 2-methylation does not universally increase anti-inflammatory and liver glycogen deposition activity of C-21 steroids. Of the steroids studied, the only cases where 2-methylation unequivocally increased the activity were in hydrocortisone, 9 α -fluorohydrocortisone, 11 β , 17 α -dihydroxyprogesterone, 9 α -fluoro-11 β , 17 α -dihydroxyprogesterone, and Δ -6-hydrocortisone. In contrast, 2-methylation produced striking decreases in anti-inflammatory and liver glycogen deposition effects of cortisone, fluorocortisone and dehydrocorticosterone. In the case of 11-ketoprogesterone no depression of activity was seen since the parent compound was essentially inactive on the above tests. In the cases of corticosterone, fluorocorticosterone, 11 β -hydroxyprogesterone, and fluoro-11 β -hydroxyprogesterone, 2-methylation did not alter the activities in either direction.

The augmentation of anti-inflammatory and liver glycogen deposition activities seen with 2-methylation is a different phenomenon than that obtained with 9 α -halogenation. In

TABLE II. Summary of Results of 2-Methylation on Glucocorticoid and Anti-Inflammatory Activity of Various C-21 Steroids.

Parent compound	No.	Activity ($\times F$)		2-Methyl analog No.	Activity ($\times F$)	
		Glycogen	Anti-inflammatory		Glycogen	Anti-inflammatory
Hydrocortisone		1	1	I	5.8	3.5
9 α -Fluorohydrocortisone acetate	II	12.6	7	III	38	9
11 β ,17 α -Dihydroxyprogesterone	IV	.1	.05	V	1.1	.3
6-Dehydrohydrocortisone	VI	1		VII	2.7	
9 α -Fluoro-11 β ,17 α -dihydroxyprogesterone	XXIII		.7	XXIV		2.5
Cortisone	VIII	.6	.4	IX	<.4	<.07
9 α -Fluorocortisone acetate		6*		XVI	.4	
11-Dehydrocorticosterone acetate	X	.4	.2	XI	<.1	<.1
11-Ketoprogesterone	XII	<.4	<.07	XIII	<.3	<.1
11 β -Hydroxyprogesterone	XIV	<.4	<.07	XV	10	<.1
Corticosterone acetate	XVII	.8	.3	XVIII	.5	.3
9 α -Fluorocorticosterone acetate	XIX		3.5	XX		2.4
9 α -Fluoro-11 β -hydroxyprogesterone	XXI		.2	XXII		.2

* Fried, J., *Annals N. Y. Acad. Sci.*, 1955, v61, 573.

all cases of published data on 9 α -halogenation of corticoids, the analogues are more potent than the parent compounds(1,2,3). The changes in activity accompanying 2-methylation of C-21 steroids described here may not be seen in other biological activities than the two reported since preliminary data on these compounds indicate that different structure-function relationships appear to occur in the case of salt retention and pituitary ACTH inhibition.

The relationships described in this series of compounds show the danger of predicting biological activity for analogues of compounds of known activity. The experience with the potency augmenting effects of 9 α -halogenation and 1,2-unsaturation of cortisone and hydrocortisone would have led to the prediction that since 2-methylhydrocortisone was more active than hydrocortisone, the same relationship would hold between 2-methylcortisone and cortisone. Such a prediction would obviously have been wrong.

Summary. The anti-inflammatory and glycogen deposition activities of some C-21 steroids and their 2-methyl analogues have been described. The 2-methyl analogues were more potent than the parent compound in the cases of hydrocortisone, 9 α -fluorohydrocortisone, 6-dehydrohydrocortisone, 11 β ,

17 α -dihydroxyprogesterone and 9 α -fluoro-11 β , 17 α -dihydroxyprogesterone. In the cases of 11 β -hydroxyprogesterone, corticosterone, 9 α -fluorocorticosterone, 9 α -fluoro-11 β -hydroxyprogesterone and 11-ketoprogesterone the 2-methyl did not potentiate the activity. The 2-methyl analogues of cortisone, 9 α -fluorocortisone and 11-dehydrocorticosterone were less effective than the parent steroid.

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Elimination of I^{131} Labelled Homologous and Heterologous Serum Proteins From Blood of Various Species.*† (22930)

WILLIAM O. WEIGLE.† (Introduced by F. J. Dixon)

Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

The tracing of I^{131} labelled proteins has been used extensively to determine the rates of elimination or half-lives of serum proteins in various species. This technic has proven to be reliable, giving data comparable to those obtained with passively transferred, internally labelled proteins(1-3) and proteins traced by immunochemical determinants(4-5). Dixon and co-workers using the I^{131} technic to study the half-lives of homologous globulins(6) and albumins(7) in the circulation have shown 1) that the half-lives of homologous serum proteins vary widely from species to species and 2) that these half-lives can be correlated with the metabolic rate of the host. On the basis of observations in the rabbit it was believed that rates of elimination of homologous serum proteins and rates of non-immune elimination of heterologous serum proteins, prior to appearance of antibody were similar, suggesting that the metabolic characteristics of the host determined the rates of all non-immune protein catabolism(8). However, the present observations in other species indicate that this situation does not hold generally.

Materials and methods. The serum proteins used in this study are listed in the accompanying table. These proteins were trace labelled with I^{131} (I^*) according to the method of Talmage *et al.*(9). Final I^* protein preparations contained less than one atom of iodine per molecule of protein and 99% of the radioactivity was protein bound as determined by trichloroacetic acid precipitation. The following animals were used: 72 guinea pigs weighing approximately 500 g, 19 albino rats weighing between 170 and 200 g

and 75 rabbits weighing between 2 and 2.5 kg. The guinea pigs and rats were injected by intracardiac puncture and through the tail veins respectively, with 7.5 mg protein/kilo body weight, whereas the rabbits were injected in the ear veins with between 15 and 30 mg of protein/kilo body weight. The animals were bled at 1-6 day intervals beginning 2 or more days after the injection. Approximately 0.5 ml of guinea pig or rat blood was collected in tubes containing potassium oxalate and heparin. Three tenths ml aliquots of the whole blood were counted in a well type gamma counter and the counts converted to percent of injected I^* protein remaining in the total blood volume, using an arbitrary correction factor for blood volume of 8% of the body weight. Rabbits were bled from the ear veins and the counts in the sera converted to percent of injected I^* protein remaining in the total blood volume. Half-lives of the rates of disappearance of I^* protein from the blood were obtained by graphic analyses. These analyses were based on the exponential elimination of the proteins which followed equilibration between intravascular and extravascular fluid spaces. To determine the effect, if any, of amount of serum protein injected on the half-life, guinea pigs and rabbits were injected with varying amounts of I^* BSA or I^* BGG and the half-lives were determined. Four groups of 4 guinea pigs each were injected with either 0.5, 7.5, 25 or 100 mg I^* BSA/kilo body weight. Two groups of rabbits (21 animals in each group) were injected with I^* BGG. One received 1 mg of the protein/rabbit and the other 100 mg/rabbit.

Results. The half-lives of all homologous and heterologous proteins used in this study plus some half-lives obtained from the observations of others are given in the accompanying table. In the guinea pig homologous globulin half-life was twice that of homolo-

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TABLE I. Half-Lives of Homologous and Heterologous Serum Proteins.

Animal	Protein	Investigator	Method of fractionation	Avg half-life in days
Guinea pig	A GPG (P)	Author	Ethanol	5.4 $\pm$.3 [†]
	GPGG	Dixon <i>et al.</i> (6)	Ammonium sulfate	5.4 $\pm$.9
	GP SA (P)	Author	Ethanol	2.8 $\pm$.2
	GP SA	"	Ammonium sulfate	2.6 $\pm$.2
	B RGG (P)	"	Ethanol	5.1 $\pm$.2
	RGG (A)	"	"	4.8 $\pm$.4
	RGG	Melcher & Masouredis(5)	Ammonium sulfate	4.5 - 4.7
	BGG (A)	Author	Ethanol	1.8 $\pm$.1
	HGG (L)	"	"	1.9 $\pm$.2
	C RSA (P)	"	"	2.3 $\pm$.1
	RSA (A)	"	"	2.2 $\pm$.1
Rat	BSA (P)	"	"	2.3 $\pm$.1
	A Rat SA	Feldman*	Ammonium sulfate	1.9 - 2.2
	Rat GG	Author	<i>Idem</i>	5.5 $\pm$.6
	B RGG (P)	"	Ethanol	6.7 $\pm$.7
	BGG	Pruzansky & Axelrod†	"	3.0
	C RSA (P)	Author	"	1.1 $\pm$.1
	BSA (P)	"	"	1.3 $\pm$.1
Rabbit	A RGG (A)	Dixon <i>et al.</i> (6)	"	4.6 $\pm$.8
	RGG	<i>Idem</i>	Ammonium sulfate	5.7 $\pm$ 1.2
	RSA (A)	Dixon, Maurer & Deichmiller(7)	Ethanol	5.7 $\pm$.3
	B BGG (A)	Author	"	2.2 $\pm$.3
	HGG (L)	"	"	3.0 $\pm$.4
	GPG (P)	"	"	1.6 $\pm$.3
	C HSA (A)	"	"	4.1 $\pm$.4
	BSA (A)	"	"	4.3 $\pm$.6
Mouse	A Mouse GG	Dixon <i>et al.</i> (6)	Ammonium sulfate	1.9
	" SA	Dixon, Maurer & Deichmiller(7)	Electrophoresis	1.2
	B RGG	Masouredis, Melcher & Shimkin(10)	Ammonium sulfate	5.3 $\pm$.2
	RGG	Dixon, Maurer & Weigle(11)	<i>Idem</i>	5.1
	BGG (A)	Deichmiller*	Ethanol	1.5
	C HSA (L)	Melcher, Masouredis & Shimkin(12)	"	1.5 $\pm$.1

BSA = bovine serum albumin, BGG = bovine gamma globulin, HSA = human serum albumin, HGG = human gamma globulin, RSA = rabbit serum albumin, RGG = rabbit gamma globulin, GP SA = guinea pig serum albumin, GPGG = guinea pig gamma globulin, GPG = guinea pig globulin; (A) = Armour and Co., (P) = Pentex Incorp., (L) = Lederle Laboratories.

* Unpublished data.

† Personal communication.

‡ $\pm$ stand. dev.

gous albumin. Heterologous globulin half-lives varied considerably while those of heterologous albumins were similar. In the rat homologous globulin was eliminated much more slowly than homologous albumin. The half-life of RGG was longer and the half-life of BGG shorter than that of homologous globulin, while the heterologous albumin half-lives were shorter than that of homologous albumin. In the rabbits the half-life of homologous albumin was approximately the same as that of homologous globulin, the heterologous albumin half-lives were somewhat shorter than that of the homologous albumin, and the heterologous globulin half-lives were consid-

erably shorter than that of homologous globulin. I*BSA was eliminated from the guinea pig at the same rate regardless of whether 0.5, 7.5, 25 or 100 mg of the protein were injected/kilo body weight. Also, the non-immune elimination of I*BGG in rabbit was the same regardless of whether 1 mg or 100 mg of I*BGG were injected.

Discussion. Three generalities can be made concerning the elimination of homologous and heterologous serum proteins from the blood of guinea pigs, rats and rabbits. First, there is no consistent relationship between the rates of elimination of homologous albumin and homologous globulin in various

species. In the guinea pig and rat as in the mouse homologous globulin half-lives are significantly longer than the albumin half-lives while in the rabbit, as in man, cow and dog the homologous albumin and globulin half-lives are similar(7). *Second*, no constant relationship exists between the rates at which homologous and heterologous gamma globulins are eliminated. In some instances heterologous gamma globulins are eliminated 2-3 times faster than the homologous gamma globulin and in other instances they are eliminated 2-3 times slower. On the other hand, the relative rates of homologous and heterologous albumin elimination are similar in all the species studied. In each species the half-life of homologous albumin is somewhat longer (20-50%) than the half-lives of heterologous albumins. *Third*, there is no consistent relationship between the rates at which various heterologous albumins or globulins are eliminated in a given species nor between the rates of elimination of a given protein in different species. The half-lives of different heterologous proteins may vary as much as 6-fold in a given species, and the half-lives of a single protein may differ more than 3-fold from one species to another.

The half-life of a protein in the circulation of a given species is probably dependent upon many factors among which are: the metabolic rate of the host, which correlates well with the half-lives of most homologous proteins, and the physical-chemical properties of the protein which would determine its physiological suitability (ability to circulate in the body fluids) and metabolic suitability (susceptibility to the host's enzymes) in the particular host. As appears to be the case, a given protein does not get similar physiological and metabolic acceptance in all heterologous species nor do different heterologous proteins in a given host. It is of interest that the physiological and metabolic suitability of a given heterologous serum protein in a particular species may have no relationship to its immunologic properties in that species. Proteins which appear most suitable physiologically may be extremely antigenic in the same species and vice versa(13).

RGG is unique in its ability to persist in the circulation of foreign hosts. Other heterologous proteins are eliminated from the guinea pig, rat and mouse 2-6 times as rapidly as RGG. In the mouse and rat RGG is eliminated even more slowly than are homologous serum proteins. RGG differs from the gamma globulin of other species not only in this *in vivo* behavior, but also in its N-terminal amino acid composition(14,15,16). Whether or not this chemical difference is related to the ability of RGG to persist in foreign species cannot be answered at the present time. In order to investigate the possibility that the *in vivo* behavior of RGG was the result of non-specific resistance to enzymatic hydrolysis, both I*RGG and I*BGG were incubated with various concentrations of trypsin and the percent of I* liberated from the protein was determined. Trypsin hydrolysed both of the proteins at approximately the same degree indicating that RGG possessed no particular resistance to *in vitro* hydrolysis by trypsin. Whatever the properties of RGG which enable it to circulate in foreign hosts they do not influence the behavior of other proteins in the same animal. When I*BSA and I*BGG were injected into guinea pigs, along with 1 ml of rabbit serum, the labelled proteins were eliminated at the same rates as when they were injected alone.

McFarlane(2) reported that the elimination of methanol fractionated homologous albumin, in the rabbit, was 30% faster than sodium sulfate precipitated albumin. Dixon *et al.*(6) also reported a shorter half-life in the rabbit for ethanol fractionated RGG than for ammonium sulfate precipitated RGG. This difference in rate of elimination between salt and alcohol fractionated serum proteins, however, is not evident in the guinea pig (table). The difference between the half-lives of alcohol and salt fractionated serum proteins, which appears to exist in the rabbit but not in the guinea pig, may be associated with either species difference or with technical factors in the preparation of the particular materials studied.

Summary. The rates at which heterologous serum proteins are eliminated from the blood

of guinea pigs, rabbits, rats and mice prior to the immune response may differ greatly from rates of elimination of homologous serum proteins. While elimination of homologous proteins, particularly albumins, appears to be related primarily to the metabolic rate of the host, elimination of heterologous proteins apparently reflects, in addition, the physiological and metabolic suitability of the protein to the particular host. Rabbit gamma globulin is unique in that it persists in foreign hosts as long or longer than do the homologous proteins. The half-lives of serum proteins in guinea pig are independent of the methods used for their separation (alcohol and ammonium sulfate fractionation).

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Viral Agents Oncolytic for Human Tumors in Heterologous Host. Oncolytic Effect of Coxsackie B Viruses.* (22931)

R. G. SUSKIND, R. J. HUEBNER, W. P. ROWE, AND R. LOVE

U. S. Department of Health, Education, and Welfare, N.I.H., National Institute of Allergy and Infectious Diseases, and National Cancer Inst., Bethesda, Md.

Ability of many viral agents of low pathogenicity for man to proliferate in and destroy neoplastic human cells in tissue culture, has intensified the search for agents that produce such an effect *in vivo* (1-5). Several viruses produced oncolytic effects in animal tumors (6,7) and particular attention has been given to their effects on tumors of ascites and lymphoma types (8,9). The possibility of adapting a virus to multiply and later to become oncolytic in a tumor system which it initially failed to infect, has also been demonstrated

(10,11). However, search for agents which are oncolytic for cancer in man thus far has made only very limited progress (12-14), possibly for want of adequate method of screening and assaying virus effects directly on human cancer cells *in vivo*. The high degree of cell and species specificity of most mammalian viruses makes it, *a priori*, unlikely that an animal tumor system will provide more than a model of specific virus-tumor relationships. Likewise, it is unwarranted to assume that behavior of a virus in tissue culture is necessarily a guide to its behavior on similar cells *in vivo* (8,15). However, a combination of the two approaches, transplantation of tissue culture grown human tumor cells into animals, might come closer to providing a practical method for *in vivo* study

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of viruses in human cancer tissue. Such a technic, of transplanting human carcinomata into irradiated and/or cortisone treated animals, has been developed by Toolan(16-17) and shown applicable for this purpose (18,19). Unfortunately, use of operative human tumor specimens injected subcutaneously in rats and into cheek pouches of hamsters, has not led, so far, to regular supply of tumor-bearing animals suitable for carrying out controlled experiments in adequate numbers. In the present study, a variation of this technic has been employed to establish on a large scale, relatively uniform transplants of human carcinomata from stable, tissue culture cell lines (HeLa and KB). The purpose of this report is to show (a) the application of this method to selection and adaptation of viral agents oncolytic for solid tumors of human cell origin, and, (b) to report in some detail the behavior of one of these agents.

Materials and methods. Irradiation: Weanling albino rats, Osborne-Mendel or Sprague-Dawley strains, weighing 40-60 g, were irradiated as follows: A single dose of 350 r was administered from two 200 KV tubes, one above and one below, at a distance of 54 cm, at 125 r/min, with 15 ma and 150 ohms, using .25 copper and .55 aluminum filters. An alternative procedure, using high voltage irradiation, was used in a few experiments where 425 r were given from a 2.5 million KV tube at 155 r/min, with a target distance of 150 cm. Cortisone acetate (Merck) 3 mg was injected subcutaneously on 2nd and again on 4th day after irradiation. Antibiotics were given prophylactically to all rats to minimize irradiation mortality. Penicillin 12,500 units, and streptomycin, 12.5 mg, were inoculated subcutaneously periodically, especially during 1st and 2nd week after irradiation. Chloramphenicol 0.6 mg/ml was added to drinking water during many experiments. Inoculum: HeLa cells[†] were grown in 32 oz. Blake bottles in medium consisting of 10% human serum in Eagle's basal medium(20). The cells were scraped from the flasks, pooled, centrifuged at 300 rpm for 10 minutes, and

made into suspensions in Eagle's basal medium. This suspension was slowly agitated with magnetic stirrer, and inoculated intraperitoneally into rats on 4th or 5th day after irradiation. Each rat received 1 ml of cell suspension containing approximately 3-5 million cells, about $\frac{2}{3}$ of cellular contents of 1 Blake bottle. Virus: The 4520 strain of Coxsackie B3, obtained from a case of pleurodynia in 1951(21), was studied intensively. The initial inoculum consisted of 10% suckling mouse carcass suspension of 8th suckling mouse passage. Titrations were performed in tissue culture tubes of monkey kidney cells[‡] using 2 tubes per 10-fold dilution. Titration end point was considered the highest dilution which produced cytopathogenic effects or from which virus was recovered on passage. Neutralization procedures were carried out as described for the adenovirus group (2nd procedure) against 100 tissue culture doses(22). Virus inoculation was performed when growth of tumor was confirmed by abdominal palpation, about 9th day after implantation. Inocula were either virus-containing tumor suspensions, or HeLa cell or monkey kidney tissue culture passages thereof. These were injected routinely by intraperitoneal route, using 0.5-1 ml; intravenous injection through tail vein was also employed. Tumor suspensions were prepared as follows. Animals were sacrificed with chloroform, the tumors weighed and made into 10% or 20% suspensions in medium 199 (24) by mincing 3 minutes in chilled Waring blender. All suspensions were frozen and thawed at least once, and clarified by centrifugation at 3000 rpm for 25 minutes. Tumor suspensions were inoculated as 10-fold ultracentrifugal concentrates in several instances. At autopsy, representative portions of tumor were removed for histological examination, fixed overnight in 10% formol-sublimate, and stained with hematoxylin-eosin.[§] Growth of tumor was followed by frequent abdominal palpation, and finally by grading the tumor

[†] Obtained from Microbiological Associates, Inc., Bethesda, Md.

[§] In some cases, tissues were stained with Van Gieson; resorcin-fuchsin, and Barrett stain(23).

[‡] Seed cultures were obtained from Microbiological Associates, Inc., Bethesda, Md.



FIG. 1. Gross appearance of HeLa-tumor 6 days after intraperitoneal inoculation of 6 tissue culture log doses of Coxsackie B₁ passed through 6 previous rat-HeLa tumors plus 3 additional passages in monkey kidney tissue culture; comparison with an uninoculated control tumor; HeLa cells were implanted 15 days prior to autopsy.

at autopsy as follows: 1. abundant tumor: numerous pearly-grey, grape-like nodules scattered throughout omentum and mesentery; histologically, many islands of tumor cells with variable but usually inconspicuous evidence of necrosis (Fig. 1, 2). 2. no recognizable tumor: an irregularly shaped mass with areas of opaque white necrosis or fibro-

sis, in which no intact tumor cells could be detected microscopically (Fig. 1,3). 3. extensive necrosis and/or fibrosis: intermediate classification between 1 and 2, in which degeneration was obvious on gross and histological observation, but some intact tumor could still be demonstrated.

Results. Production of tumor: Palpable

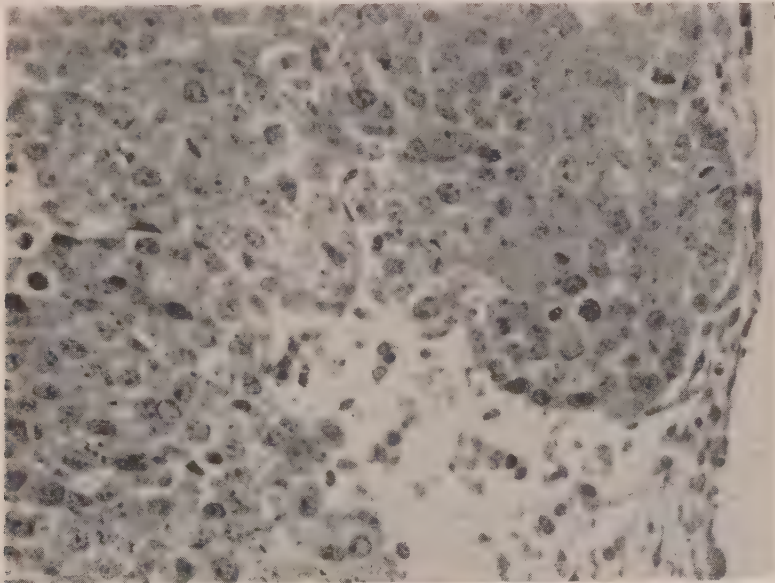


FIG. 2. Histological appearance of rat-HeLa tumor 12 days after implantation. Control tumor for Fig. 4, Barrett stain. 290 X.

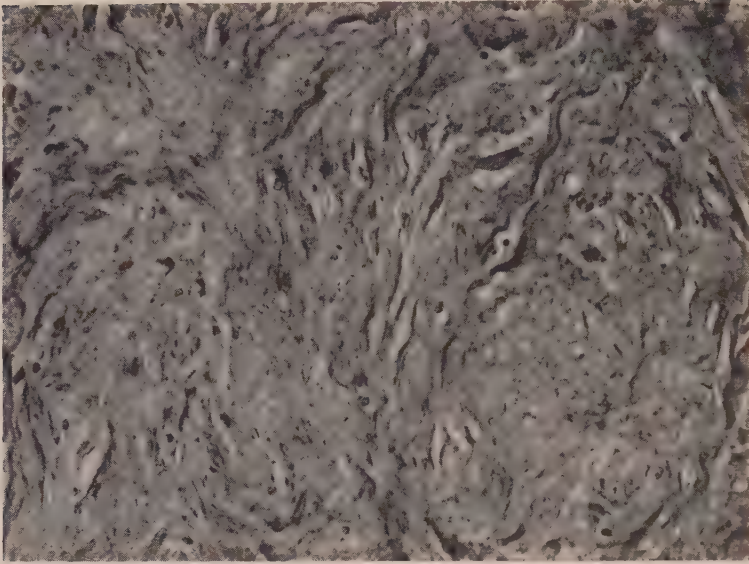


FIG. 3. End stage of tumor regression following intraperitoneal inoculation of Coxsackie B₃ passed through 6 rat-HeLa tumors. Van Gieson; resorcin-fuchsin. 290X.

tumor nodules 0.25 to 1 cm diameter were found in 93% (506/534) of rats surviving effects of whole body irradiation, at 7 to 9 days after cell inoculation. The presence of tumor was verified at autopsy in a total of 602/644 (93.6%) of rats inoculated. The tumors grew rapidly between the 7th and 18th days after implantation and their size

could be readily estimated by palpation. The median weight of 65 tumors at 14 and 15 days was 13 g; at 17 and 18 days, the median weight of 41 tumors was 17.8 g. They appeared as pearly-grey, glistening solitary or conglomerate botryoid masses in omentum, mesentery, pancreas, and occasionally spreading over peritoneal surface of diaphragm, in-

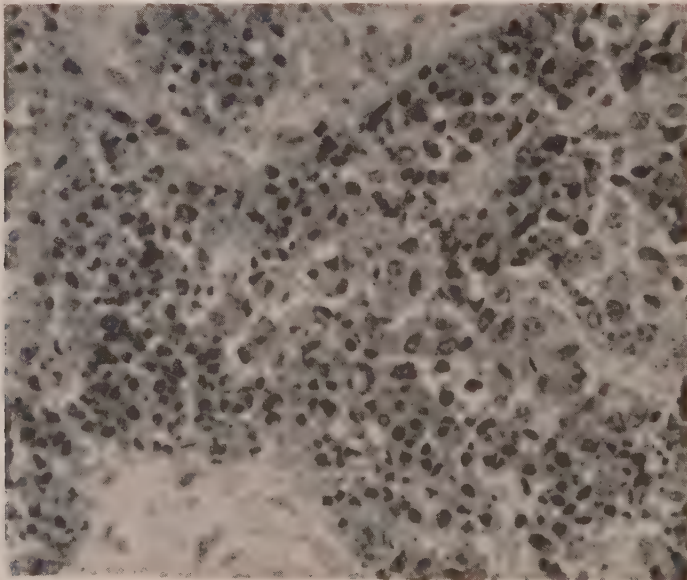
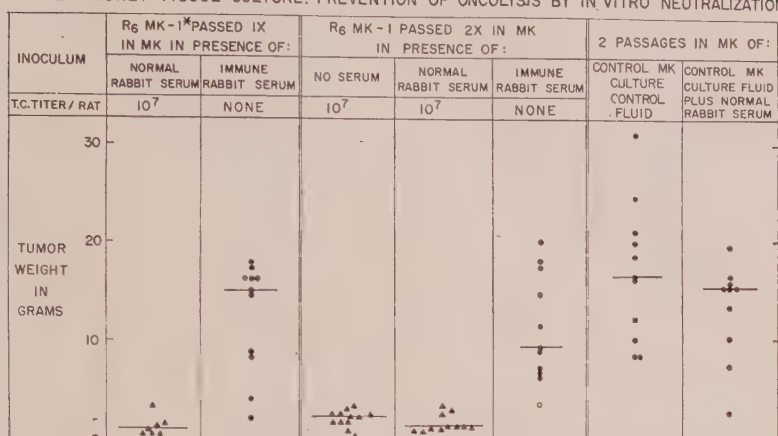


FIG. 4. Peripheral type necrosis seen 2 days following intraperitoneal inoculation of Coxsackie B₃ passed through 6 rat-HeLa tumor passages. Uninoculated control tumor in Fig. 2. 290 X.

ONCOLYTIC EFFECT OF COXSACKIE B₃ VIRUS AFTER 6 RAT PASSAGES AND PASSAGE IN MONKEY KIDNEY TISSUE CULTURE. PREVENTION OF ONCOLYSIS BY IN VITRO NEUTRALIZATION

LEGEND: ▲ - COMPLETE REGRESSION, ○ - MARKED NECROSIS OR FIBROSIS, ● - "NORMAL" TUMOR, — - MEDIAN WT. OF GROUP, * - FIRST PASSAGE IN MONKEY TISSUE CULTURE FOLLOWING 6 RAT PASSAGES

FIG. 5. See text.

testine, liver, or stomach (Fig. 1). Discrete hemorrhages and opaque white and occasionally hemorrhagic areas of necrosis were seen in some tumor nodules.

Microscopically, in first few days following implantation, the tumors consisted of multiple nodules of cells separated and surrounded by delicate strands of areolar connective tissue (Fig. 2). Mitotic figures were abundant. Small areas of coagulation necrosis were present in center of some nodules and occasional isolated degenerated tumor cells were seen. Beginning about 2 weeks after implantation, necrosis of tumor cells became more widespread, and occasionally, minute areas of necrosis appeared at periphery of nodules. Necrotic changes were accompanied or followed by some inflammatory reaction, characterized by presence of macrophages, neutrophilic granulocytes and fibroblasts around and within the nodules. Many animals with massive tumors died around this time, often in a state of dyspnoea, and sometimes apparently from late effects of irradiation and secondary infection. Animals surviving beyond 3 weeks showed more widespread necrosis, phagocytosis of tumor cells, and proliferation of fibrous tissue. Five weeks after implantation, no recognizable tumor was found in 24 survivors out

of original group of 40 rats. The overall mortality during the first three weeks after tumor implantation was 25% (216/814).^{||}

Screening for virus growth: Attempts have been made to carry a number of viruses in serial passage through tumor bearing rats. The following viruses could not be recovered after 1 or 2 passages: adenoviruses (APC) types 6 through 12, and Bertha; Coxsackie A9, and ECHO type 8. Adenovirus types 2, 3, and 4 were carried through 3, 5, and 2 serial passages respectively; no oncolytic effects were observed. Vaccinia, ECHO type 7, and vesicular stomatitis, types New Jersey and Indiana, were carried without difficulty through 4 to 6 serial passages, without production of oncolytic effects.

Coxsackie B viruses in rat-HeLa tumors: Coxsackie B₁ was passed without difficulty through 19 serial rat passages. Coxsackie B₃ was selected for more intensive study and has been carried through 22 consecutive rat passages. Up to 10⁹ tissue culture doses of virus were recovered/g tumor, varying with time of harvest and state of tumor degeneration.

^{||} Similar tumors were established on a large scale with equal success, using Eagle's KB cell(25) as inoculum (obtained from Microbiological Assoc., Bethesda, Md.)

TABLE I. Incidence of Tumor Degeneration in Infected and Uninfected Rat-HeLa Tumors as Seen in 11 Consecutive Experiments; Comparison of Gross and Histological Appearance. Inoculum consisted of Coxsackie B₃ virus passed through 4 to 8 rat-HeLa tumor passages; autopsies were performed 5 to 9 days after infection.

Gross appearance	Infected tumors				Histological appearance				Non-infected control tumors			
	Some tumor cells; severe necrosis and fibrosis			Total gross observ.	Abundant tumor cells			Some tumor cells; severe necrosis and fibrosis			Total gross observ.	
	Abundant tumor cells	No tumor cells	Not examined		Abundant tumor cells	No tumor cells	Not examined	Abundant tumor cells	No tumor cells	Not examined		
Abundant tumor	2	1	0	0	3/162	1.9	55	0	0	38	93/106	87.7
Some tumor + severe degeneration	0	2	4	7	13/162	8.0	2	3	1	3	9/106	8.5
No tumor	0	8	80	58	146/162	90.1	0	2	2	0	4/106	3.8
Total histological observations	2/97 2.1%	11/97 11.3%	84/97 86.6%				57/65 87.8%	5/65 7.7%	3/65 4.6%			

TABLE II. Growth and Recovery of Coxsackie B₃ from Tumor-Bearing and Non-Tumor-Bearing Rats Inoculated Intraperitoneally with 7 Logs of Virus. (Titers are expressed in log doses/g oriental mass, each figure representing 1 rat-HeLa tumor; figures with † and ‡ represent pools of tumors.)

Rats	Inoculum	Time after inoculation										
		30 min.	1 day	2 day	3 day	4 day	5 day	7 day	9 day	10 day	11 day	13 day
		10 ⁴ , 10 ⁵	10 ⁷ , 10 ⁸	10 ⁸ , 10 ⁹	10 ⁸ , 10 ⁹	10 ⁸ , 10 ⁹	10 ⁷ , 10 ⁸	10 ⁷ , 10 ⁸	10 ⁸ , 10 ⁹	10 ⁸ , 10 ⁹	0, 10 ⁷	0, 10 ⁷
Tumor bearing	R _c MK ₃											
	Suckling mouse susp.	10 ⁶	10 ⁶	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁸	0, 10 ⁷	0, 10 ⁷
Non-tumor bearing	R _c MK ₃											
	Suckling mouse susp.	10 ⁵	10 ⁵	0	0	0	0	0	0	0	0	0

² Coxsackie B₃ (suckling mouse suspension) passed through 6 rat-HeLa tumors and 3 monkey kidney tissue culture passages.
† Pool of 2 rats.
‡ Pool of 3 rats.

Oncolytic effects of Coxsackie B₃. In the 1st 4 rat passages, there was inconclusive evidence that viral infection influenced growth of tumors; however, massive necrosis was produced in 98.1% of tumors inoculated with virus from 4th through 8th rat-HeLa tumor passage, resulting in the absence of tumor cells in 86.6% of those examined histologically (Table I). In uninoculated control tumors, grossly similar changes were seen in 12.3%. Comparison of virus-infected tumors with controls revealed a striking difference in amount and distribution of necrosis. Between 1st and 5th days after infection, there was conspicuous degeneration of tumor cells beginning around periphery of nodules and rapidly extending inwards (Fig. 4). During this period, only a very rare degenerated cell was observed around periphery of nodules in control tumors, and degeneration, when present, was almost entirely confined to center of lobules. Necrosis of tumor cells was followed by invasion by macrophages and neutrophilic granulocytes and fibroblastic proliferation. Occasional giant cells and scanty lymphocytes, plasma cells, mast cells, and eosinophils were present. Finally, tumor nodules were replaced by dense fibrous tissue indistinguishable from that observed in control tumors 4-5 weeks after implantation (Fig. 3). Thus, during first 5 days after inoculation, necrosis spread centripetally throughout the virus infected tumors, but was virtually absent in controls. During succeeding 10-12 days, almost all virus infected tumors were completely necrotic while intact tumor cells were abundant in control tumors; only small fringes of peripheral necrosis were seen in some uninfected tumors at this period. As shown in Table I, the state of regression, as evaluated on histologic grounds, correlated very well with the independent results of gross inspection. Oncolysis and marked reduction of tumor weights were observed following both intraperitoneal and intravenous inoculation. In a representative experiment rats were inoculated with 10⁶ tissue culture dose of 6th rat-HeLa passed Coxsackie B₃ virus. Eight of 8 inoculated intraperitoneally, and 7 of 7 inoculated intravenously,

showed only scar tissue with no evidence of tumor cells, whereas 8 of 9 uninfected control rats and 7 of 8 rats inoculated with ground, frozen and thawed suspension of healthy control tumor showed abundant tumor. Monkey kidney passage of the above virus inoculum also totally destroyed tumor in 8 of 8 rats. Frozen and thawed suspensions of regressed control tumors also produced no destructive effects in other experiments. Growth of virus and destruction of tumor has also been produced by feeding the virus inoculum.

Prevention of virus effects: Oncolytic effects were prevented by heating the virus to 56°C for 45 minutes and by neutralization with Coxsackie B₃ antiserum. *In vitro* neutralization was demonstrated by experiment shown in Fig. 5. The first monkey kidney tissue culture passage of virus passed through 6 rat-HeLa tumors was carried for 2 additional monkey kidney passages in 3 parallel series, one with no serum, one with a 1:20 dilution of normal rabbit serum, and one with a 1:20 dilution of Coxsackie B₃ antiserum. This serum was prepared by immunization of rabbits with mouse passage virus and had a neutralizing antibody titer of 1:80. Fluids were harvested and passage was made when virus control tubes showed complete cytopathogenic effects; no cytopathogenic changes were seen in cultures containing immune serum. Culture fluids containing virus without serum, and with normal rabbit serum, produced complete oncolysis, while fluids from passages with immune serum produced no effect. Also, fluid from serum control tubes (containing no virus) did not affect the tumor. Monkey kidney cell suspension inoculated as a further control in a subsequent experiment, failed to produce evident gross or microscopic changes. Inhibition of oncolysis was also demonstrated in a passive immunization experiment. Rats were inoculated subcutaneously, 2 hours before, and 2 days after virus challenge, with 1 ml of Coxsackie B₃ antiserum, produced in adult mice with virus grown in suckling mice. One ml of virus inoculum, consisting of 4 log doses of virus of 4th rat-HeLa tumor passage mixed with 0.2

TABLE III. Comparison of Oncolytic Properties of Coxsackie B₃ after Rat-HeLa Tumor Passage *In Vivo* as Compared with Passage in Tissue Culture. (Starting virus material was suckling mouse suspension, 8th passage.)

Exp. No.	Day of autopsy post inoc.	Passages of inoculum	T.C. dose/rat	Abundant tumor	Some tumor + severe degeneration	No tumor
32	6	HL ₁	10 ⁶	5	2	1
		R ₆ HL ₁	10 ⁶	0	0	8
		Uninoc.		8	1	0
43	6	MK ₃	10 ⁷	9	1	2
		R ₆ MK ₃	10 ⁷	0	1	10
		Uninoc.		7	1	1
47	7	HL ₆	10 ⁷	4	1	1
		R ₆ MK ₃	10 ⁷	0	1	9
		Uninoc.		1	2	0
63	7	MK ₄	10 ⁷	5	1	0
		R ₆ MK ₃	10 ⁸	0	0	6*
		HL ₆	10 ⁷	3	0	1
		R ₆ HL ₆	10 ⁷	0	2	4
		Uninoc.		4	0	0

R₆ = 6 rat-HeLa tumor passages. HL₆ = 6 HeLa-tissue culture passages. MK₃ = 3 monkey kidney tissue culture passages.

* Gross observation only.

ml of mouse immune serum, was inoculated intraperitoneally. A control group was inoculated in the same way with normal serum; all groups were sacrificed on the 6th day. Abundant tumor was found in 7 of 11 rats which were passively immunized, severe degeneration was seen in 2, and no tumor in one. In the virus control group without serum, of 13 rats, 11 showed no tumor, and 2, severe degeneration. The mouse immune serum, however, had a neutralizing titer of only 1:5. Virus was recovered from pooled tumors in each group, indicating that low-titered immune serum used produced incomplete *in vivo* neutralization.

Growth of virus in tumor tissue: Table II illustrates growth of virus in tumor tissue in representative experiments. Maximum titers were obtained during the first 5 days in the experiment using 6th rat-HeLa tumor passed virus, which coincided with the period during which destruction of tumor occurred. Microscopic evidence of extensive necrosis was first seen on 2nd day after inoculation. The decline and gradual disappearance of virus could not be related to antibody production, for when sera of these rats were tested at a dilution of 1:5 for neutralizing antibody, only 1 rat serum, taken on seventh day, showed neutralization. It appears probable that de-

struction of tumor tissue was responsible for the decline in titer of virus. Growth of suckling mouse propagated virus in tumor bearing rats was also compared with its growth in irradiated, Cortisone-treated rats not inoculated with HeLa cells (Table II). Virus was recovered in high titer from the tumor tissue until the seventh day, but not after the first day, in omentum and pancreas of non-tumor bearing rats. Present evidence indicates that Coxsackie B₃ virus does not proliferate in tissue other than tumor in this system. When recovered from brain and muscle tissue in tumor bearing rats, it has been in lower concentrations than in tumor or serum.

Adaptation. Present evidence, gathered in experiments designed primarily for other purposes, suggests that repeated rat-HeLa tumor passage produced enhancement of oncolytic activity (Table III). Suckling mouse grown virus was used for initiating both rat-HeLa tumor and tissue culture passages. It is apparent that tumor destruction was more severe after *in vivo* passage in rat-HeLa tumor than after passage in tissue culture of either monkey kidney or HeLa cells. Experiments comparing virus passed through 13 rat-HeLa tissue culture passages have confirmed this difference between oncolytic properties of

virus passed *in vivo* and *in vitro*. The effect of repeated mouse passage has not been determined. Present evidence also indicates that rat-HeLa tumor passed Coxsackie B₃ virus also has an oncolytic effect on rat-KB tumors. Thus, Coxsackie B₃ virus, passed through 13 rat-HeLa passages (titer 10⁻⁶), when injected into rats with KB tumor, was followed by disappearance of tumor in 10/11 rats in 6 days, 1/11 showing severe degeneration. Healthy tumor was seen in 9/10 uninoculated controls. Inoculations of the same quantity of virus which had been passed through 13 HeLa tissue culture passages was followed by severe degenerative changes in 2/8 KB tumors, but abundant tumor was seen in 6/8. Consistent destruction of HeLa cell tumors occurred after 5th rat-HeLa passage of Coxsackie B₁, after 4th passage of B₂, and 7th passage of Coxsackie B₅.

Discussion. The high degree of specificity for certain cell types shown by many viral agents, and the demonstrated possibility of altering such specificities by serial passage through different host systems, provides a theoretical background for supposing that some of these agents could be selected or adapted to produce oncolytic effects specifically in neoplastic cells(11,12). Our previous report (14) described the use of adenoviruses (APC) in experimental virotherapy of carcinoma of the cervix. In these studies, as well as those of Southam and Moore(12,13), the *in vitro* cytopathogenic effects of viral agents for cell types in tissue culture had little or no relationship to the specific cytologic effects, if any, produced in the same tissue *in vivo*. In furthering the search for agents oncolytic for human tumors, a practical laboratory method for comparing *in vivo* with *in vitro* viral effects seems most desirable. The previous work of others(16-19,26), especially that reported by Toolan and her associates, indicated that practical methods could be achieved for this purpose. The present investigations were, therefore, undertaken in an effort to develop an investigative tool which would be useful in selecting and adapting viral agents with oncolytic properties *in vivo*.

Our technic seems to serve this purpose by

providing comparatively uniform, large carcinomata of human origin, capable of growing some prevalent viruses, namely, Coxsackie viruses, adenoviruses, ECHO viruses, and vaccinia virus. Tumor to tumor passage readily resulted in adapting at least 4 Coxsackie B viruses to produce profound oncolytic effects. A similar passage of one of these strains through the same line in tissue culture failed to produce such adaptation. This observation, as well as the fact that only some agents cytopathogenic in HeLa tissue culture grew in the transplant, and others grew without oncolytic activity, supports evidence available from studies in ascites tumors(8) that the oncolytic capacity of a virus *in vitro* does not necessarily correspond to its effect *in vivo*.

The mechanism of oncolysis in this system has not been determined; host factors may contribute to tumor destruction initiated by the virus(27), and further investigation of the mechanism of cell destruction is in progress. The application of viruses having oncolytic properties in animal-grown human cell tumors, to virotherapy of human cancer, depends, of course, on clinical trials and perhaps additional selection or adaptation by passages through the human host.

Summary. 1. The Toolan technic for growing cancer tissue in a heterologous host was applied to growth of tissue culture propagated HeLa and KB cells in the peritoneal cavity of rat. This method resulted in rapid development of large healthy explants of carcinoma on peritoneal surfaces, particularly of omentum. Ninety-three % of rats inoculated developed comparatively uniform tumor masses, thus allowing for adequately controlled studies of virus growth and oncolytic effects. 2. A preliminary survey of adenovirus, ECHO, and Coxsackie agents in rat-HeLa tumor system produced 3 types of reaction: 1. failure of some agents to grow. 2. growth of virus without oncolytic activity, and, 3. growth of virus with evidence of slight or incomplete oncolytic effects. Four Coxsackie agents, types B₁, B₂, B₃, and B₅, showed initial evidence of growth, and on serial passage through rat-HeLa tumors, in-

creased capacity for destroying solid carcinomata *in vivo*, producing characteristic histological changes. The oncolytic Cocksackie B₃ virus produced by rat-HeLa tumor passage *in vivo*, compared to the same strain passed in HeLa cells grown in tissue culture, revealed marked differences in oncolytic capacity. Use of this method illustrates the possibility of virus adaptation by tumor to tumor passage; that this principle has been successfully employed to destroy solid tumor transplants of human origin, suggests its possible application for future clinical studies.

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Utilization of Vitamin A and Carotene by Selenium Poisoned Rats.* (22932)

J. G. BIERI, C. J. POLLARD, AND R. R. CARDENAS, JR.†

Laboratory of Nutrition and Endocrinology Natl. Inst. of Arthritis and Metabolic Diseases,
P.H.S., U. S. Department of Health, Education and Welfare, Bethesda, Md.

A previous study has shown that aqueous dispersions of carotene given intravenously to rats are readily converted to vit. A(1). In further experiments designed to obtain information about the site of conversion of carotene injected intravenously, attempts were

made to block the reticuloendothelial system and also to damage the liver.

Methods. Weanling male rats of Holtzman strain were placed on synthetic, vit. A-free diet as follows: purified casein 18, salts 4(2), sucrose 74, cottonseed oil‡ 2, vit. mixture in sucrose 2(3). Half of the animals in each experiment received 12

* Part of this work was done at University of Texas Medical Branch, Galveston.

† Present address: Department of Microbiology, University of Texas, Austin.

‡ Wesson oil.

TABLE I. Storage of Vitamin A by Normal and Selenium Poisoned Rats (Mean Values $\pm$ Stand. Error).

Exp.	No. rats	Days on selenium	Avg wt	Supplement	Time interval, hr*	Liver + kidney			
						Carotene		Vit. A	
						Total, μ g	μ g/g	Total, μ g	μ g/g
1	5	0	147 $\pm$ 3.6	100 μ g caro-	65	0		26.4 $\pm$ 1.3	3.9 $\pm$.3
	5	8	129 $\pm$ 3.0	tene orally	65	0		13.6 $\pm$ 2.7	2.3 $\pm$.4
2	6	0	100 $\pm$ 4.0	95 μ g caro-	6	28.2 $\pm$ 1.2	6.0 $\pm$.2	9.0 $\pm$ 1.5	2.6 $\pm$.4
	6	22	96.1 $\pm$ 3.0	tene inj.	6	26.3 $\pm$.9	6.0 $\pm$.3	5.8 $\pm$.2	1.1 $\pm$.1
3	3	0	84.7 $\pm$ 6.0	68 μ g caro-	6	20.8 $\pm$.7	4.7 $\pm$.4	7.7 $\pm$.5	2.2 $\pm$.2
	4	14	80.0 $\pm$ 3.5	tene inj.	6	20.5 $\pm$.9	4.2 $\pm$.2	2.6 $\pm$.8	1.0 $\pm$.3
4	5	0	97.6 $\pm$ 5.6	100 μ g vit. A	24	0		31.6 $\pm$ 3.7	5.8 $\pm$ 1.0
	8	7	80.0 $\pm$ 2.5	orally	24	0		13.8 $\pm$ 1.7	2.9 $\pm$.3
5	5	0	83.0 $\pm$ 1.6	100 μ g vit. A	24	0		32.9 $\pm$ 2.6	7.5 $\pm$.5
	8	7	77.1 $\pm$ 2.1	+ 0.3 mg vit. E orally	24	0		12.9 $\pm$ 1.4	3.2 $\pm$.4

* Interval animal was permitted to survive after giving the supplement.

ppm of selenium, as sodium selenite, in the diet. Due to differences in maternal diet, some groups became depleted of vit. A in 17-21 days while others required 30-35 days. Selenium did not affect rate of depletion. Food intake of control rats was restricted to the amount consumed by the selenium-fed group. Animals were caged singly in air-conditioned room at $75 \pm 1^\circ\text{F}$. Supplements of crystalline carotene (85% β -, 15% α -) dispersed in water with 20% Tween 40 or of vit. A acetate dissolved in cottonseed oil[†] were administered by tail vein or by stomach tube when vit. A deficiency was apparent. The rats were sacrificed 6 hours after injection, or 33-65 hours after stomach-tubing. In the latter groups, selenium was removed from diet 18 hours prior to giving carotene or vit. A supplement. All animals were fasted at least 8 hours prior to death. Vit. A in combined kidneys and livers was determined with antimony trichloride on an aliquot of the hexane extract of the unsaponifiable fraction, using correction for color due to carotene. Analyses of deficient rats revealed no detectable vit. A in livers and kidneys. Rats depleted of vit. A as described above were injected with trypan blue or carbon tetrachloride prior to administering carotene, and the tissues analyzed for vit. A. The conditions are given in Table II.

Results. In Table I it can be seen that utilization of carotene, as measured by tissue storage of vit. A, was impaired in rats fed

selenium. Regardless of whether carotene was given orally (Exp. 1) or intravenously (Exp. 2 and 3), the amount of vit. A was only about one half that of control animals. To determine if selenium might diminish utilization of pre-formed vit. A, 2 groups were dosed orally with vit. A acetate (Exp. 4). Tissues of selenium-treated animals again contained less than one-half as much vit. A as normal animals. In Exp. 5, dl, α -tocopherol was incorporated into the vit. A supplement to protect the vitamin against possible oxidation in the digestive tract. This failed to prevent decrease in storage of vitamin in selenium-fed animals.

Discussion. Rosenfeld and Beath(4) found that in selenium fed sheep a marked drop in vit. A content of blood and liver occurred, even though the diet contained an adequate amount of carotene. Ascorbic acid in these tissues was also significantly depressed by selenium.

In the present study, it is possible that selenium impaired intestinal absorption of vit. A. In view of the relatively efficient utilization of orally administered carotene, however, it appears that this factor was not of great significance. The effect of selenium on vit. A may be explicable by two different mechanisms, first, that seleniferous tissues destroy vit. A, but not carotene, and secondly, that poisoned liver may have decreased capacity for holding the vitamin. It has been shown *in vitro* that ascorbic acid is oxidized by se-

TABLE II. Carotene Utilization by Rats Injected with Trypan Blue or Carbon Tetrachloride.*

Treatment	No. rats	Avg wt	Carotene supplement	Vit. A, $\mu\text{g/g}$	
				Liver	Kidneys
None	4	113 $\pm$ 6.5	120 μg , I.V.	2.7 $\pm$.2	1.9 $\pm$.1
Trypan blue†	6	107 $\pm$ 2.5	<i>Idem</i>	2.1 $\pm$.6	2.3 $\pm$.4
CCl ₄ ‡	6	113 $\pm$ 2.7	"	2.4 $\pm$.1	2.1 $\pm$.3

* All rats were killed 6 hr after inj. with carotene.

† Inj. I.P. with 1 ml of 2% trypan blue; inj. with carotene 24 hr later.

‡ Inj. subcut. with 0.2 ml CCl₄ in cottonseed oil (50% w/vol) for 3 consecutive days; inj. with carotene on fourth day.

lenium salts and this mechanism was proposed as the cause of decreased ascorbic acid in livers from selenium-poisoned rats(5). However, we have not observed increased destruction of vit. A incubated in rat blood containing added sodium selenite (unpublished observation).

In support of the second possibility, that selenium alters storage capacity of the liver, the recent study of Krishnamurthy and Ganguly(6) may be quoted. They found that rats injected with India ink decreased liver storage of vit. A ester, but not of vit. A alcohol. This would support the theory previously proposed by these workers(7) that different proteins may specifically bind various carotenoids in blood. The fact that storage of carotene was not affected in our study, whereas storage of vit. A was, would lend further support to this theory. However, it should be pointed out that the 2 types of poisons (selenium and India ink) damage different structures in livers. In contrast to the results of Krishnamurthy and Ganguly, impairment of reticulo-endothelial system by trypan blue had no effect on carotene utilization in our study (Table II).

The observation that rats with selenium-poisoned livers could still convert carotene injected intravenously supports our previous

conclusion(1) that this organ is not of significance in conversion of pro-vitamin. Recently, McGillivray *et al.*(8) have shown that injected carotene can be transformed to vit. A in the rat even when the entire liver has been removed.

Summary. Rats fed sodium selenite had impaired utilization of carotene given orally or intravenously. Storage of pre-formed vit. A was reduced, but not storage of injected carotene. Poisoning with trypan blue or carbon tetrachloride did not affect carotene conversion.

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A Method for Assay of Insulin Using Alloxan Diabetic Hypophysectomized Mouse. (22933)

E. ANDERSON, F. WHERRY, R. W. BATES AND J. CORNFIELD

Section on Endocrinology, N. I. Arthritis and Metabolic Diseases and Division of Research Services, N. I. H., Bethesda, Md.

Interest in the development of methods for the assay of micro quantities of insulin stems from the desire to measure the amount of insulin in circulating blood. According to Waters and Best(1), if one estimates the concentration of insulin in blood on the basis of the daily dose of insulin required by patients who have had a total pancreatectomy, which is about 30 units, the amount of insulin would be no more than 200 microunits (μ U) per ml of blood. By another method of estimation using insulin I¹³¹ Berson *et al.*(2) arrive at a value of 65 μ U per ml body fluids. Two methods have been described for the micro assay of insulin, one using an *in vitro* technic and the other an *in vivo* technic. By the *in vitro* technic the isolated rat diaphragm is incubated in glucose buffer solution and the effects of added insulin upon the utilization of glucose and the deposition of glycogen in the muscle are measured. Willibrands and Groen(3) have prepared a critical review of this type of assay based on the reports of several workers (Stadie and Zapp(4); Krah and Park(5); Perlmutter, Weisenfeld and Mufson(6); Groen, *et al.*(7); Vallance-Owen and Herlock(8); and Randle(9)). *In vivo* technics which measure the fall in blood sugar in response to insulin in animals which have been rendered exceedingly sensitive to insulin have been described by Hemmingsen, Nielsen and Nielsen(10), using the adrenalectomized rat; by Gellhorn, Feldman and Allen(11), using the adrenodemodulated hypophysectomized rat; by Anderson, Lindner and Sutton(12), using the adrenodemodulated alloxan diabetic hypophysectomized rat; and by Bornstein(13), using the adrenalectomized, hypophysectomized alloxan diabetic rat. Recently, Yenermen *et al.*(14) described a method using the alloxan diabetic hypophysectomized mouse, which purported to measure accurately amounts of insulin as

low as three microunits. In an attempt to repeat the work described in the latter report the present authors were unable to reproduce the results. The method which will be described here uses, also, the alloxan diabetic hypophysectomized mouse. Amounts of insulin as low as 250 microunits can be measured if the insulin is administered intravenously or 1000 microunits if it is injected intraperitoneally.

Methods. Male mice (CAF₁) aged 3-4 months and weighing 25-30 g are used. The animals are made diabetic with alloxan. For this procedure the mice are placed in a well ventilated incubator set at 30°C for 2 hours without food or water. Alloxan monohydrate, 1.0% solution in physiological saline, is injected into the tail vein in a dose of 70 mg/kg body weight. In a group of 60 mice given this dose of alloxan 48 hours previously, the non-fasting blood sugar level of 56 mice was over 300 mg %, averaging 390 mg %. Forty-eight hours after alloxan administration the mice are put on daily subcutaneous injections of 2 units protamine zinc insulin (PZI) to restore them to a healthy state in preparation for hypophysectomy. A week is allowed to elapse between the alloxan administration and this operation. The day before operation regular insulin is substituted for PZI and no insulin is given on day of operation. Since hypophysectomy renders the mouse sensitive to insulin almost immediately, in order to avoid fatal hypoglycemia, it is well to give the animals food by stomach tube (egg-milk mixture) an hour before operation in case traces of insulin are still present in the tissues. The technic of hypophysectomy for the mouse is essentially the same as that originally described by Smith(15) for the rat. Veterinary nembutal (Abbott) is used as the anaesthetic, the intraperitoneal dose is 0.065 mg/g body weight. The best survival rates

after hypophysectomy are obtained if the animals are given 6% glucose in a solution of 0.5% NaCl in place of tap water to drink and fresh whole wheat bread and crisp kale twice a day. Overcrowding of animals in the cages is avoided. The cages are kept in a ventilated incubator at 30°C. If the animals are used for assay not oftener than once a week, they may survive for as many as 6 assays. The animals remain in the incubator during the test, except for the few minutes when they are removed for injection or the taking of blood samples. Blood glucose is determined on 0.01 ml of blood taken from the tip of the tail. The method of Park and Johnson(16) is used. The starting blood sugar values in a group of 15 to 30 mice, which is the number used in one day's assay, may vary from 100 to 350 mg %. The practice is to use 1.0 ml of the supernatant fluid after protein precipitation for all the samples and to bring the color of the most concentrated solutions into a satisfactory range for the spectrophotometer reading by diluting with blank solution. Crystalline glucagon-free insulin[†] is used for the standard. One stock solution has served as the insulin standard during the entire period in which the data of this report have been collected. The stock solution of insulin is prepared by dissolving 2 mg of insulin in 5 ml of 0.2% phenol solution containing a drop of N acetic acid. This solution contains 10 U/ml. Aliquots are diluted with sterile saline to the desired concentration for injection upon the day of the experiment. The standard solution is stored in the refrigerator at 4°C. *Intravenous method.* It has been found useful to have at least 2 trained people carry out this procedure, since the exact timing of each step is critical for this method. An example of the routine procedure in our laboratory is given here. Two groups each of 12 to 15 test mice are used for one assay; the first group is started at 9:00 a. m. and the second group at 9:45 a. m., while the first group is being processed. 9:00 a. m. 25 mg glucose in 1.0 ml saline are injected subcutaneously into mice in

Group I. The animals are placed in clean cages without food or water and returned at once to the incubator. 9:45 a. m. The animals are placed in individual mouse holders, given Nos. #1 to #15, respectively, and returned to the incubator. 10:00 a. m. Test dose of insulin in 0.1 ml saline is injected intravenously into mouse #1. The rest of the mice are injected one minute apart, in numerical order. 10:05 a. m.[‡] Initial blood specimen from mouse #1 and from a mouse every minute thereafter in numerical order. Blood proteins are precipitated immediately according to the method for the blood sugar determination. 10:25 a. m. Final blood specimen from mouse #1, etc. The second group is carried through in the same way.

The injections consist of saline and 3 or 4 dose levels of insulin with 6 test animals for each. In most experiments the data from one or 2 animals have to be discarded for such reasons as: failure to deliver the dose intravenously, failure to obtain a blood sample, or because the blood glucose levels are too low for quantitative determination.

Intraperitoneal method. The number of test animals used in this procedure is 24 to 30. For identification purposes the mice are separated into groups of 6 to a cage and each is given a distinguishing mark, such as: pink head, pink back, etc. 9:00 a. m. 25 mg glucose in 1.0 ml saline are injected subcutaneously. The order in which the animals are injected is maintained for subsequent procedures. The animals are without food or water and are returned to the incubator as soon as injected. 10:00 a. m. Initial blood specimen from a mouse every minute in numerical order. 10:01 a. m. Test dose of insulin in 0.1 ml saline injected intraperitoneally into mouse #1, etc. 10:31 a. m. Final blood specimen from mouse #1, etc.

Results. A summary of the blood sugar response to different doses of intravenously administered insulin is presented in Fig. 1, and for intraperitoneally administered in-

[†] We wish to thank Dr. Otto K. Behrens, Eli Lilly and Co., for the glucagon-free insulin.

[‡] The reason for taking the initial blood specimen 5 minutes after insulin injection is to avoid the necessity of correcting for dilution caused by intravenous injection.

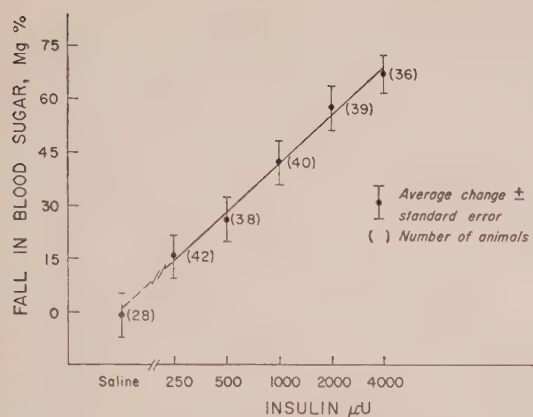


FIG. 1. Change in blood sugar for different doses of insulin, intravenous method.

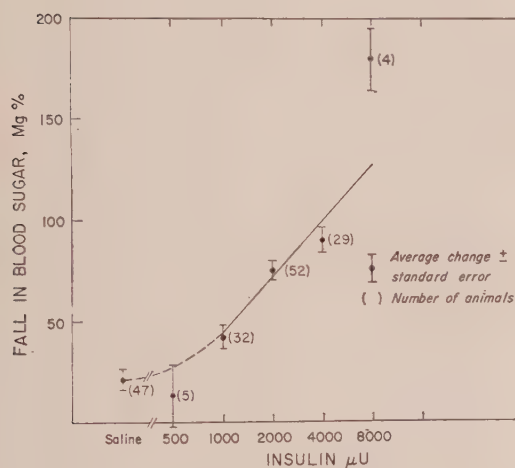


FIG. 2. Change in blood sugar for different doses of insulin, intraperitoneal method.

sulin in Fig. 2. Each observed point is an average of the falls in blood sugar for the number of mice indicated in parentheses. The standard error of each mean is indicated by the vertical bar. For the intravenous route the biological response is clearly a linear function of log dose in the range of 250 to 4000 μ U. The least amount that can be measured is in the neighborhood of 250 μ U, since response to any smaller amount will not differ appreciably from response to saline. For the intraperitoneal assay administration of saline is accompanied by a significant fall in blood sugar. In consequence the least detectable amount of insulin is higher than for the in-

travenous assay, something between 500 and 1000 μ U.

These results are obtained by combining experiments done at a number of different times and results for animals used once or several times. In order to distinguish between these two kinds of experiments as well as to indicate the nature of the variation from experiment to experiment we show the separate results for each experiment for the intravenous assay in Table I. The number of animals used in any one experiment is small and the results in consequence somewhat variable. When appraised against the variation in response from animal to animal treated alike within an experiment, however, the variation between experiments is no greater than might be expected by chance. We have therefore pooled all experiments separately for intravenous and intraperitoneal assays, but have maintained the distinction between results obtained with first and subsequent use. (There is some difficulty with survival of animals during the summer months. Assays carried out at that time have been discarded.)

A summary of these results is shown in Table II. It will be noted that for both the intravenous and the intraperitoneal methods steeper slopes are obtained on second and subsequent use than on first use. Whether this is because of some effect of use, such as accustoming the animal to handling, or related to the time elapsing after hypophysectomy, we are unable to say. The steeper slopes after repeated use are counter balanced to some extent by higher standard deviations, so that indices of inherent precision, λ , are much the same on first as on subsequent use.

The value of λ for the intravenous assay, 0.7, is somewhat higher than that reported for other animal assays of insulin (Bliss and Cattell, 17). This simply means that to attain any required level of precision more observations are required. Thus, if one wishes to estimate the potency of an unknown preparation the number of animals required to attain any desired precision is given by the formula $n = (2 \lambda / s_M)^2$, where s_M is the selected standard error of log potency. If s_M is chosen as 0.301, corresponding to a standard

TABLE I. Intravenous Method, Average Fall during 20 Min. in Blood Sugar (mg %) by Dose Level and Number of Previous Times Assay Animal Was Used. A negative value stands for a rise in blood sugar.

Date of exp.	Saline	Insulin dose (μ U)				
		250	500	1000	2000	4000
First use						
11/14/55	-25 (1)*		-19 (1)	2 (2)	64 (2)	
16	8 (3)		26 (4)	31 (6)	38 (5)	
18		14 (4)			45 (3)	77 (3)
25	26 (2)	10 (2)	41 (1)	45 (1)		
28	7 (4)	5 (5)	35 (3)	36 (4)		
12/22	- 4 (1)	24 (1)	17 (3)	42 (4)		42 (2)
1/ 4/56	8 (3)	43 (3)	14 (3)	24 (2)	60 (2)	68 (2)
11	- 8 (3)	33 (5)	30 (3)	29 (4)	35 (3)	47 (6)
All exp.	4.4(17)	21.0(20)	23.0(18)	32.3(22)	45.2(15)	56.4(13)
Second or later use						
11/14/55	-15 (1)			59 (1)	32 (1)	
18		-30 (1)			68 (4)	65 (3)
21		20 (4)			52 (4)	63 (9)
23			45 (7)	59 (5)	93 (3)	125 (2)
25	-36 (2)	0 (4)	24 (4)	8 (1)		
1/ 6/56	3 (2)	4 (4)	26 (4)	73 (4)	67 (4)	51 (3)
13	2 (3)	14 (4)	16 (2)	46 (4)	78 (4)	80 (2)
17	-10 (3)	18 (5)	13 (3)	46 (3)	53 (4)	103 (4)
All exp.	-9.5(11)	10.9(22)	29.3(20)	54.2(18)	66.0(24)	75.5(23)

* No. of assay animals on which avg is based shown in parentheses.

error of twofold in the estimation of potency, 22 animals would be required, half for the standard and half for the unknown. The value of λ for the intraperitoneal assay is less than for the intravenous, but the greater sensitivity of the latter makes it the method of choice.

Summary. Two methods for the assay of small amounts of insulin are presented. Both involve using the alloxan diabetic hypophysectomized mouse. One involves measuring the fall in blood sugar in test animals over a

20 minute period after the intravenous administration of insulin, the insulin being given one hour after the subcutaneous injection of 25 mg glucose. The other method involves measuring the fall in blood sugar 30 minutes after the intraperitoneal administration of the insulin, which, as in the intravenous method, is given one hour after a 25 mg glucose meal. The intravenous method, which is the more sensitive of the two, is capable of detecting as little as 250 μ U of insulin.

TABLE II. Comparison of Precision of Intravenous and Intraperitoneal Method, First and Subsequent Use of Animals.

	Intrav. method	Intraper. method
1st use		
Stand. dev. (mg %)(σ)	23.3 $\pm$ 1.9*	33.6 $\pm$ 3.1
Slope (mg %/10-fold dilution)(b)	30.9 $\pm$ 4.6	70.2 $\pm$ 12.5
Index of precision (σ /b)(λ)	.76 $\pm$.13	.48 $\pm$.10
2nd and subsequent uses		
Stand. dev.	38.9 $\pm$ 3.0	35.9 $\pm$ 2.9
Slope/10-fold dilution	57.4 $\pm$ 7.3	97.5 $\pm$ 9.1
Index of precision	.68 $\pm$.10	.37 $\pm$.05

* Stand. error.

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Lessened Insulin Hypophosphatemia Following N-Sulfanilyl-N'-Butylurea (BZ-55 or Carbutamid).^{*} (22934)

T. S. DANOWSKI, M. H. KUNKEL, F. M. MATEER[†] AND F. A. WEIGAND

Renziehausen Foundation and Children's Hospital of Pittsburgh.

N-sulfanilyl-N'-butylurea (BZ-55 or Carbutamid) was administered to juvenile diabetics prior to insulin tolerance tests in an attempt to determine whether the amelioration of clinical diabetes reported, at a conference, with this compound can be attributed to potentiation or prolongation of insulin action.

Materials and methods. Twenty-seven juvenile diabetics, previously maintained for 3 to 5 days on short-acting insulin received *per os* 100 mg of BZ-55/kg body weight 45 minutes before start of intravenous insulin tolerance test, 0.1 u of crystalline insulin/kg given at zero time. Samples of venous blood obtained without stasis at -45, -20, 0, 30, 60, 90, 120 minutes, and in some patients at 3 and 4 hours, were analyzed for concentrations of sugar by Hagedorn-Jensen procedure. Levels of inorganic phosphorus, potassium, total carbon dioxide, chloride, and sodium in sera of venous bloods drawn between zero and 2 hour points were measured by standard procedures(1). The patients were fasted and at bed rest. Control insulin tolerance tests were made in 14 juvenile diabetics under the same circumstances save that BZ-55 was not given and blood sampling at -45 and -20 minutes was omitted.

Results. From Fig. 1 it is evident that BZ-55 neither potentiates nor prolongs the

hypoglycemic action of exogenous insulin. The mean decrements in blood sugar concentrations at 30 minutes (-52 and -36 mg % without and with BZ-55) and at 60 minutes (-77 and -69 mg % without or with BZ-55, respectively) are of the same order of magnitude in the 2 groups with p values of 0.11 and 0.56. On the other hand the decrease in serum inorganic phosphorus which accompanies the hypoglycemia is distinctly less in patients receiving BZ-55, p value of 0.02 (Fig. 1), with mean values of -0.53 and -0.80 mg % at 30 minutes. Changes in serum carbon dioxide content, potassium, chloride and sodium were not influenced by BZ-55.

Discussion. A number of possible explanations have been advanced for amelioration of diabetes mellitus in certain patients by N-sulfanilyl-N'-butylurea, including suppression of pancreatic alpha cell activity(2), decreased absorption from the gastro-intestinal tract (3), decreased gluconeogenesis, increased hepatic glycogenation(4), diminished glucose-6-phosphatase activity(5), inhibition of cytochrome oxidase(6), increased production of insulin(7), and inhibition of insulin destruction(8,9). Insofar as the last of these is concerned there is no evidence in our studies that BZ-55 intensifies or prolongs hypoglycemia which follows administration of insulin in these dosages to juvenile diabetics. As a matter of fact the lesser degree of hypophosphatemia is in keeping with a diminution of

^{*} Aided by grant from Department of Health.

[†] Established Investigator of Am. Heart Assn.

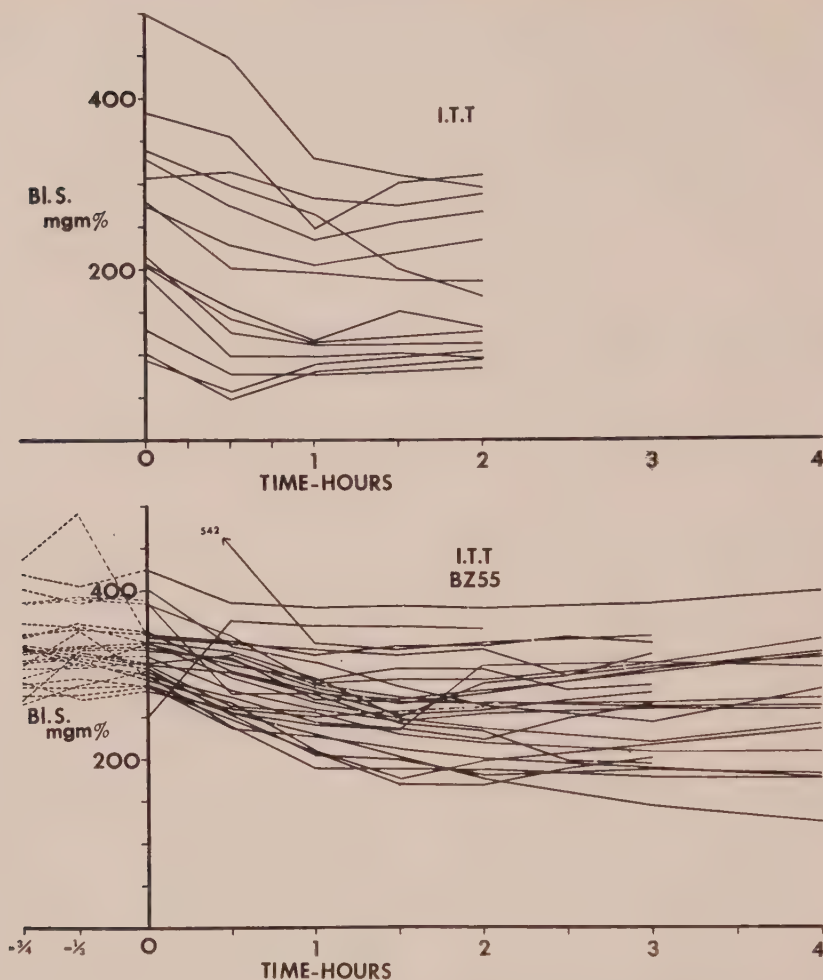


FIG. 1. Blood sugar levels in insulin tolerance tests in juvenile diabetics: effects of BZ-55. There is no evidence that BZ-55 increases hypoglycemia following intravenous administration of crystalline insulin 0.1 u/kilo of body weight at zero time (p values of 0.11 and 0.56 for the 30 and 60 minute points, respectively, in the 2 groups).

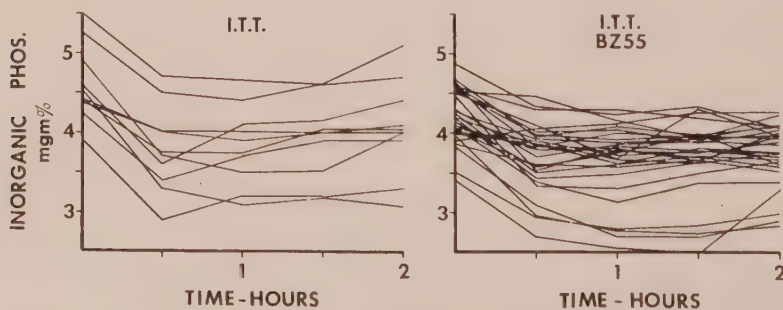


FIG. 2. Insulin tolerance tests in juvenile diabetics: serum inorganic phosphorus concentrations with and without BZ-55. Prior administration of BZ-55 decreases hypophosphatemia present at 30 minutes following intravenous insulin from -0.80 mg % in control group to -0.53 in BZ-55 group (p value of 0.02).

anaerobic glycolysis and could represent either a blockade or other loss of insulin action or result from a decrease in the glucose load. Either or both of these alternatives is compatible with decreased gluconeogenesis, enhanced glycogenation or decreased glycogenolysis as mechanisms whereby BZ-55 may lessen the insulin requirement in some diabetics.

Summary. Insulin tolerance tests in juvenile diabetics following administration of N-sulfanilyl-N'-butylurea (BZ-55 or Carbutamid) failed to show potentiation or prolongation of the usual insulin hypoglycemia and hypophosphatemia. Actually, the decrease in serum inorganic phosphorus which followed administration of insulin to juvenile diabetics was less pronounced, suggesting a diminution in anaerobic glycolysis, either as a result of interference with the usual action of insulin or as a consequence of a decreased glucose load. Both of these possibilities are compatible with decreased gluconeogenesis,

increased glycogenation, or diminished glycogenolysis as factors in the decreased insulin requirement which follows administration of this agent to certain patients with diabetes mellitus.

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Abnormal Serum Protein and Bone Destruction in Transmissible Mouse Plasma Cell Neoplasm (Multiple Myeloma). (22935)

MICHAEL POTTER,* JOHN L. FAHEY,* AND H. IRA PILGRIM†
(Introduced by L. W. Law)

National Cancer Institute, Bethesda, Md.

Plasma cell neoplasms of the C3H/HE strain mouse are rare and most often originate in an ulcerated lesion in the ileocecum. Neoplasms of this type have previously been described pathologically and one has been transplanted in this laboratory successfully through 50 transfer generations(1,2,3,4). Plasma cell neoplasms of other mouse strains have been described(5,6,7). The resemblance of these neoplasms to multiple myeloma of man has been made on the basis of microscopic pathology only. The subject of this

report is another successfully transplanted C3H strain plasma cell neoplasm of ileocecal origin (X5563), which resembles multiple myeloma of man, not only microscopically, but also by producing an abnormal serum electrophoretic pattern and multiple osteolytic lesions.

Materials and methods. 1. *Origin of tumor, serum sources and methods of transplantation.* The plasma cell neoplasm X5563 originated in a 22½-month-old female of C3H/HE/CRGL strain which had been gonadectomized at the age of 2 months. This animal developed an abdominal mass, and at autopsy there was found a large neoplasm in the wall of the ileocecum, and an enlarged

* National Institutes of Health, P.H.S., U. S. Dept. of Health, Education and Welfare.

† Department of Zoology, University of California, Berkeley.

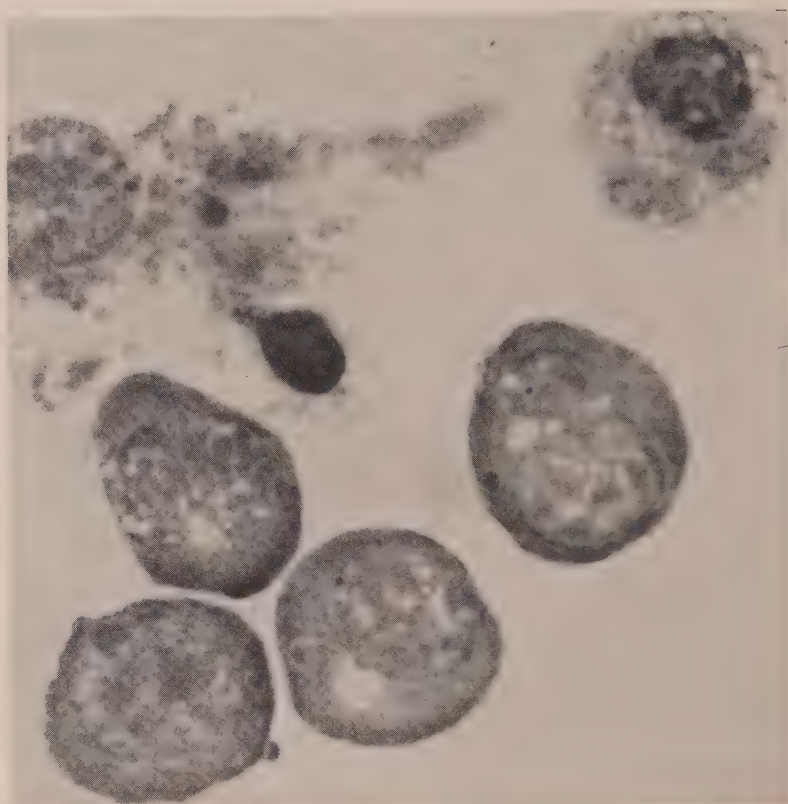


FIG. 1. Free neoplastic cells of neoplasm X5563, extruded into serous ascitic fluid which collects during intraperitoneal growth. Cytoplasm stained intensely blue. Cell in upper right part of the field is an histiocyte. Wrights' stained smear.

mesenteric node. Fragments of the ileocecal neoplasm were transplanted into subcutaneous connective tissues. Continuous subcutaneous transfer for 5 transfer generations has subsequently been maintained. Two methods of transfer have been employed; subcutaneous transfer employing 12 and 13 gauge trochars, and by intraperitoneal inoculation of homogenates prepared in Potter-Elvehjem tissue homogenizers. The subcutaneous trochar technic has proved the more desirable. Routine transfer can be carried out every 30 days. Serum for use in electrophoretic studies was obtained for the most part by cardiac puncture. Mice bearing neoplasm X5563 develop a moderate hypervolemia, and 2 to 3 ml of whole blood can be obtained by this method. 2. *Other serum sources.* Normal serum was obtained for controls from C3H/DE, C3Hf/Lw mice both derivatives of the C3H/HE strain(8). Serum was ob-

tained from C3H mice bearing spontaneous and transplanted neoplasms as follows: 1) 2 spontaneous mammary tumors designated MT-1, MT-2 (original tumor bearing mice) arising in C3H/HE females, 2) an undifferentiated lymphocytic neoplasm designated L5649 (transfer generation 16) of C3Hf/Lw origin, 3) a spontaneous thymic lymphocytic neoplasm designated L7688 (transfer generation 6) of C3H/HE origin, 4) an unusual lymphoma designated L9380 (original tumor bearing animal) of C3Hf/Lw origin, and 5) serum from various animals bearing the ascitic plasma cell neoplasm 70429 (transfer generations 30 to 55) transplanted to C3Hf/HE, C3Hf/Lw and C3H/DE mice. Serum was obtained from mice bearing various spontaneous and methylcholanthrene induced transplantable DBA/2 lymphomata: 1) P329 (transfer generation 2) P632 (transfer generation 1) both type A reticulum cell sar-

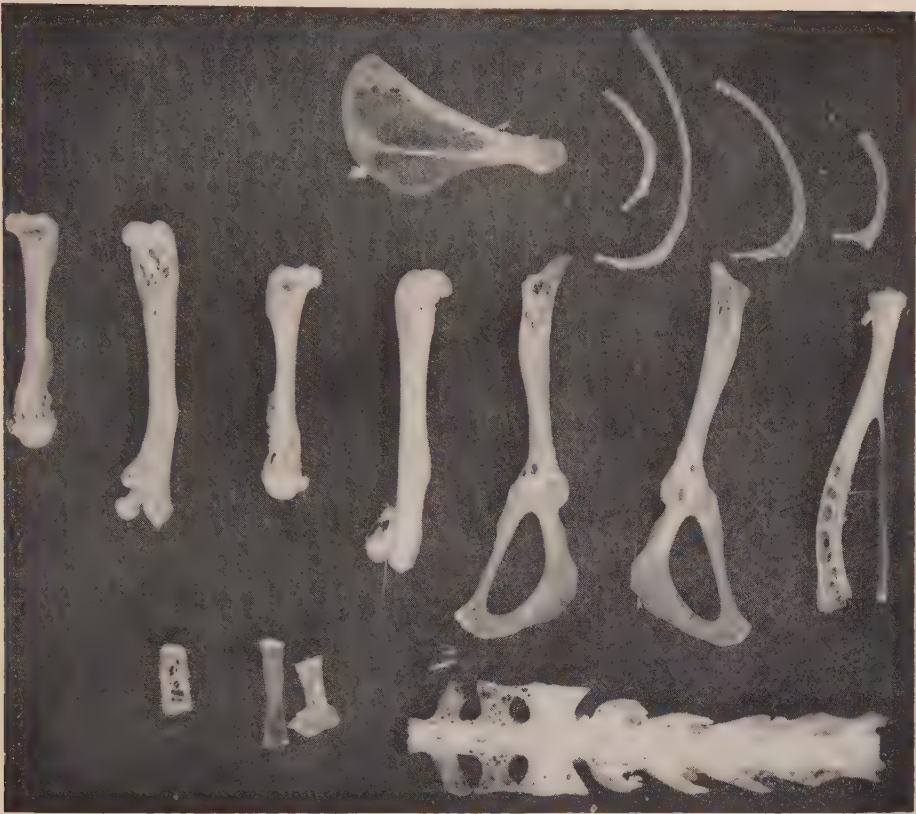


FIG. 2. Photogram of bones from a mouse bearing neoplasm X5563 for about $3\frac{1}{2}$ months. (Papain digested carcass, magnification 2.85X.)

comata(3), 2) P195 (transfer generation 7) a type B reticulum cell sarcoma(3), and 3) P353 (transfer generation 6) an undifferentiated lymphocytic neoplasm(3). 3. Mice used for maintenance of the plasma cell tumors were C3Hf/HE mice or direct derivatives of this colony C3Hf/Lw or C3H/DE. These mice were fed Derwood chow and water *ad libitum*(9). 4. Preparations of skeletons were made by papain[‡] digestion; by the method described by Searle(10). 5. Photograms[§] of skeletons were made by passing the light of photographic enlarging apparatus through the bones with exposure of negative film. 6. Pathologic sections were fixed in Tellyesniczky's fluid (70% ethyl alcohol 20 parts,

formalin 2 parts, glacial acetic acid 1 part) or Zenkers' solution and stained with hematoxylin and eosin. Smears were stained with Wright's stain. 7. Paper electrophoretic determinations were made on 0.008 to 0.010 ml of serum in hanging strip cell(11) in 0.075 μ veronal buffer at pH 8.6. Eight Whatman 3 mm strips were run simultaneously in one cell for 18 hours at room temperature under constant current conditions of 6 m. amps per cell and under potential of 68 to 72 volts. The strips were stained with bromphenol blue under conditions recommended by Jencks *et al.*(12). The paper strip patterns were evaluated by transmission densitometry with the Analytrol^{||} using a cam which provided linear response for albumin dye binding. Each electrophoretic run in a given cell included one strip which contained 0.008 ml from pooled

[‡] Commercially obtained from Nutritional Biochemical Laboratories.

[§] This method was devised by Mr. Vernon E. Taylor of the N.I.H., Scientific Reports Branch.

^{||} Spinco Division, Beckman Instruments Inc., Belmont, Calif.

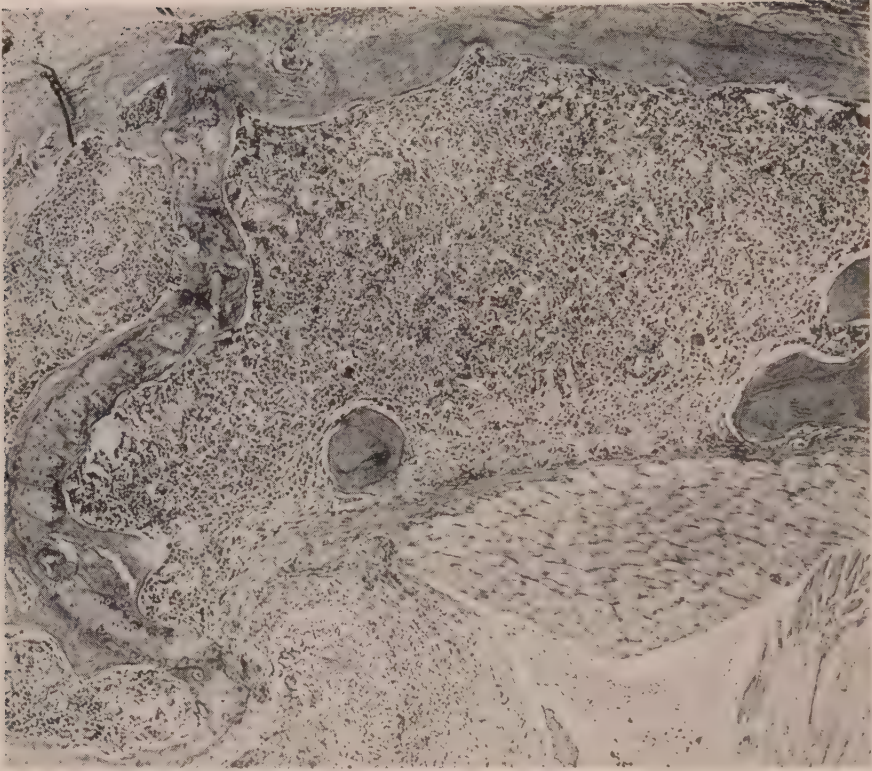


FIG. 3. Section through proximal end of humerus of mouse with multiple osteolytic lesions. Interruption of osseous tissue may be seen in lower portion of the field. Distal margin is bounded by rounded bone, proximal by an absence of bone at epiphyseal plate. H. and E. Stain magnification 70X.

human serum of known composition used as a control for all electrophoretic determinations. Migration distance (or "Mobility") of the abnormal protein was calculated relative to the movement of the albumin in each serum
$$\left(R_A = \frac{\text{mm myeloma protein moved}}{\text{mm albumin moved}} \right).$$

Total serum protein determinations were made by the biuret method according to Gornall(13).

Results. 1. Pathology. Neoplasm X5563 grows locally in subcutaneous tissues and from these tissues disseminates very late to marrow cavities. Local growth is very firm, and has a fibrous character when cut; it is reddish in color, and is moderately vascular. Liver and kidneys are not involved with infiltrations of cells as they are in lymphocytic neoplasms. Rarely, rounded masses are seen in the spleen. Regional lymph nodes in the

deep peritoneum are infiltrated. Intraperitoneal infiltrations progress from local sites, and usually accumulate in the mesentery and in the retroperitoneal regions. Intraperitoneal passage has not produced an ascitic tumor in the first two intraperitoneal transfers, though 2 to 3 ml of the slightly cloudy serous fluid does collect. This fluid contains a few cells, a normal flora of lymphocytes, mast cells and histiocytes; and tumor cells which are hyperchromatic cells of an immature type resembling plasma cells. Many of these are multilobulated and some of the smaller cells possess a spoke wheel arrangement of the chromatin.

An illustration of characteristic tumor cells found in the peritoneal fluid may be seen in Fig. 1. Such cells are unique with this neoplasm, similar cells have never been seen in other ascites lymphomata of the mouse including lymphocytic neoplasms, type A reticulum

ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS

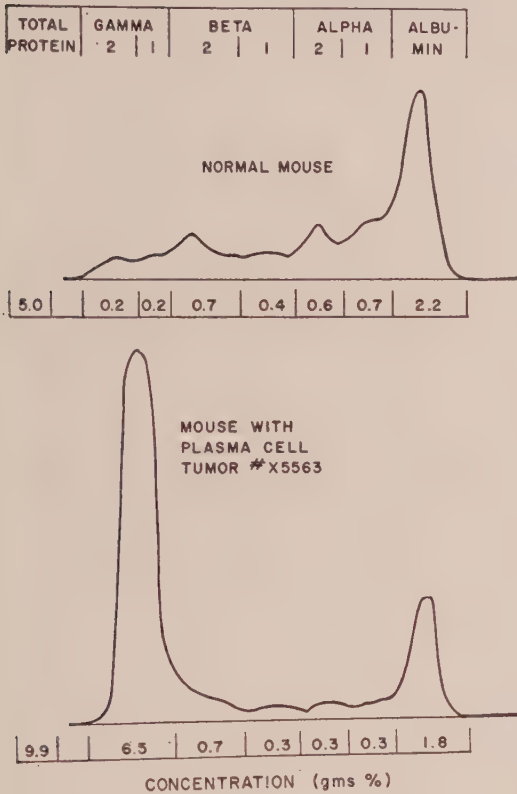


FIG. 4.

cell sarcomata, a mast cell neoplasm, or ascitic extrusions of undifferentiated lymphocytic neoplasms. Detailed tissue sections of this neoplasm will be presented by Dr. Thelma Dunn, National Cancer Institute, in a forthcoming publication.

By use of sections, and papain digested skeletons, it has been possible to study infiltration of bone marrow and subsequent bone destruction in mice bearing neoplasm X5563. In certain animals, bearing neoplasm X5563 for periods of 3 months and longer, invasion of the marrow cavity did occur, with subsequent eruption of some lesions into surrounding soft tissues. Three such sites have been observed. In 2 cases lesions were seen in the spine where paraplegia was the outcome, in one case in the sternum, and in the fourth case in the distal femur and proximal tibia where a large tumor mass was seen.

A more comprehensive view of the skeletal changes is observed in papain digested skele-

tions where multiple osteolytic lesions are seen. These are most marked in the distal femora, proximal tibiae, sacrum and lower thoracic spine, the sternum, scapula and ribs, (Fig. 2). A total of 26 skeletons of various animals bearing different age transplants were studied. Multiple osteolytic lesions similar to those shown in Fig. 2 were found in 8 of 11 mice which carried the tumor for a period of 90 days or longer. Most of these were found in animals bearing subcutaneous transplants.

Histologic section of the advanced bone lesions reveals infiltration by the neoplastic plasma cells. More precisely in areas where bone destruction is observed in tissue sections, and as can be seen from Fig. 3, there is a sudden interruption of the osseous tissue along the shaft of the bone. No osteoclastic activity is seen. The osseous edges of the lesion are rounded. These areas probably are the same as those rounded punched out areas seen in the papain digested skeletons. The marrow cavity under these lesions is filled with invading neoplastic plasma cells.

2. *Serum changes.* Serum protein analysis by zone electrophoresis revealed the presence of an abnormal protein with the mobility of a gamma globulin (Fig. 4). The abnormal serum protein was present in all animals with palpable tumors. It was not evident on examination of 28 sera from normal mice or mice bearing any of the 9 other tumors tested (Table I). The mobility of the abnormal

TABLE I. Electrophoretic Examination of Mouse Serum.

Animal and tumor type	No. tested	Abnormal protein present
<i>C₃H</i> mice		
Plasma cell tumor—X 5563	19	100%
Normal	4	0
Mammary carcinoma	2	0
Plasma cell tumor—70429	12	0
Lymphatic leukemia—67688	3	0
Lymphoma—L 5649	1	0
" L 9481	1	0
<i>DBA f/2</i>		
Lymphoma—P 195	2	0
" P 329	1	0
" P 353	1	0
" P 632	1	0

TABLE II. Serum Protein Changes Produced by Growth of Tumor X 5563.

Days after transplant	Tumor wt, mg	Serum proteins (g %)								
		Total	Normal component total	Individual components						
				A	α-1	α-2	β-1	β-2	M	γ-2
19	310	5.7	4.4	2.9	.3	.3	.2	.6	1.3	.1
33	830	7.1	4.1	2.2	.3	.4	.3	.7	3.0	.2
33	2590	9.0	3.6	2.0	.3	.3	.3	.6	5.4	.1
68	8185	11.6	3.4	1.6	.4	.4	.3	.6	8.2	.1

protein was uniform in the serum from all of the animals tested. The mobility relative to albumin (R_A) in consecutive serum samples from 13 individual tumor-bearing mice was .10 ± .01. The quantity of abnormal protein in the serum varied with the amount of tumor present. Table II illustrates the quantitative relationship of tumor to myeloma protein. The larger the tumor the greater the amount of the abnormal serum protein component. The other globulin components did not decrease, although albumin tended to fall with progression of tumor growth. These changes in the normal serum protein components are in general agreement with those previously described in tumor bearing mice(14).

Urine specimens from 4 animals with palpable tumors and the abnormal serum protein were concentrated by ultrafiltration and examined by electrophoresis. Two had no detectable protein, and the other 2 each had a single protein component with the mobility of albumin. No protein was present with a mobility the same or slightly faster than the abnormal serum protein. There was no evidence of a Bence-Jones protein by the usual heat precipitation-solubility test.

Because of the possibility that the abnormal serum proteins may be formed in the plasma cells of the tumor, a saline extract of the tumor was examined by electrophoresis. For comparison, electrophoretic analysis was also performed on serum from the same animal as well as a saline extract from a plasma cell neoplasm 70429 that is not associated with an abnormal serum protein. The results, illustrated in Fig. 5, clearly demonstrate the presence of a component in the saline extract of neoplasm X5563 that is not obtained from neoplasm 70429. The mobilities of this cellular protein and of the abnormal serum pro-

tein are the same. The demonstration of an abnormal protein in the tumor cells with electrical properties similar to the abnormal serum protein suggest that the abnormal protein of the serum may originate in the tumor cell.

Discussion. These findings reveal a mouse neoplasm (X5563) bearing a similar histologic picture to human plasma cell neoplasms that is also similar in abnormal globulin production and the pathologic predilection for

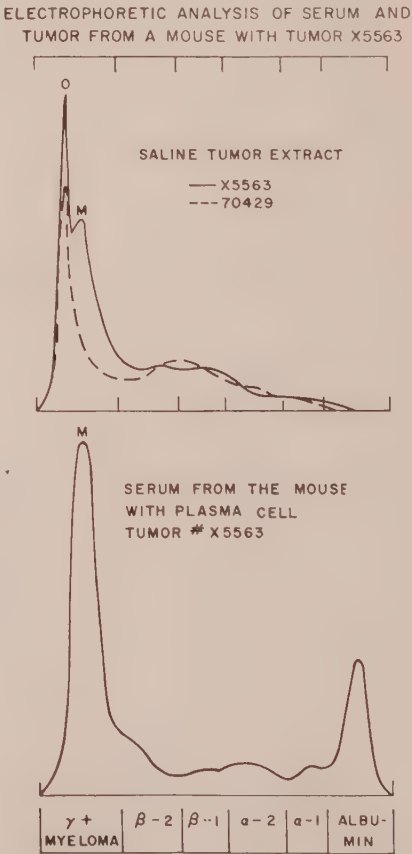


FIG. 5.

invasion of bone marrow with subsequent osteolysis and tumor formation. This triad of findings was found to be associated only with this relatively differentiated plasma cell neoplasm, and not with other lymphomata of the C3H and DBA/2 mouse, including the plasma cell neoplasm 70429 which had been transplanted over 50 transfer generations.

The concept that the plasma cells are the source of the abnormal serum protein is not new. The demonstration of a protein in the tumor cells with properties similar to the abnormal serum protein is suggestive of such a relationship. The transfer of cells to normal hosts, which later develop the abnormal serum protein, as the neoplasm grows, is again evidence to relate intimately the neoplastic plasma cell itself with abnormal serum protein production.

It is not possible at the present time to say whether the abnormal electrophoretic peak represents an abnormal amount of a normal serum component, or a protein not normally present in serum. Work is in progress to evaluate this point.

The striking uniformity of the abnormal serum protein found in the various animals bearing neoplasm X5563 would appear to differ sharply from the variety of abnormal proteins present among patients with multiple myeloma(15). However, this difference is not surprising when it is remembered that the transplants of neoplasm X5563 are all from one source and are introduced into animals of the same genetic background. In any individual human patient with multiple myeloma, the properties of the associated abnormal serum protein remain quite constant.[¶] Thus in this property, plasma neoplasm X5563 resembles an individual human myeloma. The variety among plasma cell tumors

in humans is paralleled by the difference between the two plasma cell tumors currently available in this laboratory, X5563 being associated with an abnormal serum protein and 70429 without such a protein. It is also possible that plasma cell neoplasm 70429 has lost the ability to form an abnormal serum protein in the process of laboratory transfers.

Summary. A plasma cell neoplasm X5563 of the C3H mouse has been described, which resembles human multiple myeloma, by similar histologic appearance of the neoplastic plasma cell; by similar development of osteolytic bone lesions after considerable residence in the host mouse, and by similar production of an abnormal serum globulin.

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[¶] Unpublished observations.

Synthesis of Pantoic Acid and Its Congeners by *Neurospora*. (22936)

MIYOSHI IKAWA* AND JEAN S. O'BARR. (Introduced by E. E. Snell)

Biochemical Institute, University of Texas, and Clayton Foundation for Research, Austin, Texas.

Wagner and Haddox(1) reported that the *pantothenicless* mutant (5531) of *Neurospora crassa*, when grown in aerated cultures for periods of longer than four days, synthesized in appreciable quantity what appeared to be pantothenic acid. This together with the fact that acetone-dried mycelia of the mutant could effect the synthesis of pantothenic acid from its precursors(2) was significant in biochemical genetics theory in casting doubt on the hypothesis that the mutant lacked the enzyme system for synthesizing pantothenic acid. These observations have prompted us to study the growth factors of the pantothenic acid group which are elaborated into the culture medium by a wild type and a *pantothenicless* strain of *Neurospora crassa*.

Materials and methods. *Neurospora crassa* 5256A (Emerson wild type) was grown on the medium described by Horowitz and Beadle(3). For growth of the *pantothenicless* mutant 5531A the medium was supplemented with pantothenic acid. Both wild type and mutant were obtained from Dr.

R. P. Wagner, Department of Zoology, University of Texas, Austin. Further details on the conditions of growth are given in Table II. Pantothenic acid and related compounds were determined microbiologically with *Saccharomyces carlsbergensis*(4), *Lactobacillus arabinosus*(5), *Acetobacter suboxydans*(6) at pH 5.5, *pantothenicless N. crassa* and *Lactobacillus helveticus*(7). Specificity of the response of these organisms to pantothenic acid and related compounds is summarized in Table I. The nutritional response of *N. crassa* to these compounds resembles closely that of the yeast, *Saccharomyces carlsbergensis*. Coenzyme A was determined by the arsenolysis method of Stadtman, *et al.* (15). An extract from sonically disrupted *Escherichia coli* cells served as the source of transacetylase. The bioautograph technic of Winsten and Eigen(16) was employed to differentiate related growth factors. The plates were poured with the pantothenate-free assay medium for *A. suboxydans* to which was added 2% agar. When heavily seeded with

TABLE I. Activity of Various Pantothenic Acid-Related Compounds in the Growth of *N. crassa* 5531A and Other Microorganisms.

Compound	<i>N. crassa</i> *	Activity (equivalent basis)			
		<i>A. suboxydans</i> §	<i>S. carlsbergensis</i>	<i>L. arabinosus</i>	<i>L. helveticus</i>
D-Pantothenic acid	1	1	1	1	1
D-Pantoic acid	0	1 (6)	0 (4)		
D-Pantolactone	0	0 (6)	0 (10)	0 (12)	
D-Pantothenic acid 4'-phosphate	0	0 (8,9)	0 (8)	0 (8)	
D-Pantothenyl-L-Cystine	<.001†	.1 (6)	<.02 (11)	<.02 (11)	<2 (11)
D-Pantethine	.002‡	2.8 (6)	0 (7)	.41 (7)	100 (7)
DL-Pantethine 4'-phosphate	0	5.9 (6)		.005 (13)	17 (14)
D-Pantethine 4'-phosphate	0	2.7 (6)			
Coenzyme A	0	10.8 (6)	0 (7)	0 (7)	0 (7)

Numbers in parentheses refer to literature citation.

* Based on dry wt of washed mycelium after 48 hr growth in 25 ml standing cultures.

† By assay with *S. carlsbergensis* this sample of pantothenecystine had a relative activity (pantothenic acid = 1) of 0.0006 on an equivalent basis.

‡ By assay with *S. carlsbergensis* this sample of pantethine had a relative activity (pantothenic acid = 1) of 0.0001 on an equivalent basis.

§ Comparative activities at pH 6.

|| Activity for *Saccharomyces cerevisiae*.

* Present address: Department of Biochemistry, University of California, Berkeley.

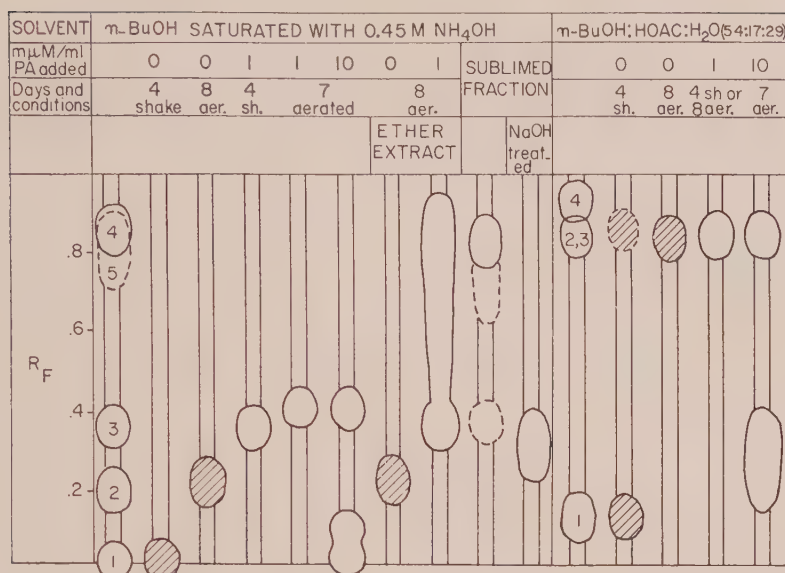


FIG. 1. Bioautographs of culture filtrates of *Neurospora* using *A. suboxydans* as the indicating organism. Shaded spots are from wild type extracts, unshaded spots from mutant 5531A extracts. Reference compounds: (1) coenzyme A, (2) pantothenic acid (PA), (3) pantoic acid, (4) pantetheine, (5) pantolactone.

a washed suspension of *A. suboxydans* in a top layer of agar, growth zones in response to topical application of active compounds were readily detected after 24 hours at 30°C.

Results. The predominant active compound for *A. suboxydans* produced by 4-day cultures of wild type *Neurospora* is coenzyme A (Fig. 1). Assuming coenzyme A to be 5.5-10 times as active as pantothenic acid for *A. suboxydans* (6,14) at pH 5.5 on the

molar basis, coenzyme A could account for 44-80% of the activity of the culture filtrate for *A. suboxydans* (Table II). By microbiological assays small amounts of pantothenic acid and pantetheine could be detected (Table II). With longer growth and aeration of the culture, coenzyme A disappeared while pantothenic acid and pantetheine increased in concentration. Bioautographs of these culture filtrates (Fig. 1) confirmed the presence

TABLE II. Assay of *Neurospora* Culture Filtrates for Various Pantothenic Acid-Related Compounds.

Growth conditions	Days	Pantothenic acid added (mμM/ml)	Pantothenic acid equiv. (mμM/ml)			LBF as pantetheine* (mμM/ml)	Co A (mμM/ml) by arsenolysis
			<i>A. suboxydans</i>	<i>S. carlsbergensis</i>	<i>L. arabinosus</i>		
Wild type	4, standing	0	24	.8	.7	<.3	
	4, shaking	0	25	1.8	1.6	.5	
	4, "	0	88	3			7
	8, aerated	0	32	6.5†		1.2	0
	8, "	0	11	3	2	1.4	
5531A	4, standing	1	20	.6	.7	<.3	
	4, shaking	1	28	.6	.5	<.3	
	8, aerated	1	35	1‡		.4	0
	7, "	1	53	.5	.2	<.1	
	7, "	10	73	10	9	.4	

* One LBF unit = 0.17 mμmoles pantetheine.

† 7 mμM/ml pantothenic acid by *N. crassa* 5531A assay.

‡ <2 " *Idem*

of pantothenic acid. Taking pantetheine (LBF) to be approximately 4 times as active as equimolar amounts of pantothenic acid at pH 5.5(14), 32-72% of the activity for *A. suboxydans* in 7-8-day aerated cultures of *Neurospora* would appear due to pantetheine and pantothenic acid. The form in which LBF occurs has not been determined; it does not appear to be pantetheine itself. Although pantothenic acid 4'-phosphate has been detected in spent *N. crassa* culture filtrates(14, 17), this compound is reportedly inactive for *A. suboxydans*(8,9) and would not add to the activity for this organism.

Culture filtrates of the *pantothenicless* mutant of *Neurospora*, grown with minimal but sufficient added pantothenic acid to give good growth (1 m μ mole per ml), showed high activity for *A. suboxydans*, but did not exhibit any coenzyme A activity (Fig. 1). The pantothenic acid content of such filtrates was equal to or slightly less than that originally added to the medium and only traces of LBF activity could be detected (Table II). Bioautographs of 4- and 7-day cultures showed only a single growth zone which corresponded to pantoic acid (Fig. 1). Tatum and Beadle (18) have reported the accumulation of pantoic acid by the *pantothenicless* mutant of *Neurospora* but without experimental details.

Fractionation of *N. Crassa* 5531A Culture Filtrates. To confirm the presence of pantoic acid a partial purification of the substance produced by the mutant was made. The mold was grown in 5 liter lots for 7-9 days with aeration. The culture medium (activity $\approx$ 1-2 m μ moles pantoic acid per mg dry wt) was filtered, the pH of the filtrate adjusted to 3, and 20 g Darco G-60 added per liter of filtrate. After stirring for 1 hour the carbon was filtered off, washed with a small amount of water, and eluted with 0.2 of the original volume of acetone containing 1% concentrated ammonia. The acetone was evaporated *in vacuo* and the residue concentrated to a point where considerable crystalline material (ammonium acid tartrate) was formed. This was filtered off. The recovery of active material at this point was 21-27%. When the adsorption and elution was done in small

test batches recoveries of the order of 50% were realized. The filtrate was adjusted to pH 9 and extracted with 3 volumes of ether. The extract contained a negligible amount of active material. The aqueous phase was acidified to pH 2, extracted 4 times with 3 volumes of ether, and the ether extract concentrated *in vacuo* to dryness. The ether extract contained 43% of the active materials in the charcoal eluate. The active material in the ether extract was chromatographed on paper. It migrated as a mixture of pantoic acid and its lactone. To an aliquot of the ether extract ($\approx$ 25,000 m μ moles pantoic acid) was added 0.5 ml 6N hydrochloric acid. The solution was taken to dryness *in vacuo* and the resulting residue sublimed at 100° and 0.02-0.03 mm for 2 hours. The sublimate showed activity equivalent to 500 m μ moles of pantoic acid before and 15,000 m μ moles of pantoic acid after sodium hydroxide saponification; the non-sublimed residue showed activity equivalent to 200 and 11,000 m μ moles of pantoic acid respectively, before and after sodium hydroxide treatment. The increase in activity results from conversion of pantoyl lactone to the salt of the free acid. The sublimate contained 170 m μ moles of pantoic acid per mg (2.5%). Bioautographs of the fractions clearly indicated the active material to be pantoic acid (Fig. 1). As a control, a sample of wild type culture filtrate from a 10-day aerated culture was adsorbed with charcoal in the same fashion, eluted, and the eluted material extracted 3 times at pH 2.0 with 7 volumes of ether. Only 12% of the active material in the eluate was extracted by the ether. When a solution of pantothenic acid containing 1 μ mole per ml was subjected to the same ether extraction procedure, 13% appeared in the extract. The ether extract from wild type *Neurospora* chromatographed as pantothenic acid (Fig. 1).

When the *pantothenicless* mutant was grown in the presence of a large excess of pantothenic acid (10 m μ moles per ml) for 7 days with aeration, no change in the pantothenic acid content of the filtrate was observed and no significant increase in LBF activity (Table II). Bioautographs of the culture filtrate in-

dicated, however, the presence of coenzyme A and other active compounds in addition to pantoic acid.

Discussion. Wagner and Guirard(19) surmised that the *pantothenicless* mutant of *Neurospora* lacked the enzyme for coupling pantoic acid and β -alanine since wild type mycelium synthesized pantothenic acid from its precursors whereas mycelium from the mutant did not. In accord with this interpretation, we have been unable to demonstrate synthesis of pantothenic acid by growing cultures of this mutant, but have demonstrated accumulation of the precursor, pantoic acid. Wagner reported, however, that acetone-dried mycelium of this mutant could effect this synthesis from the precursors(2) and that on growing the mutant for 8 days under aeration in the presence or absence of precursors a considerable amount of pantothenic acid was synthesized as judged by assay with *L. arabinosus* and bioautographs(1). It was concluded, therefore, that the coupling enzyme was present, but ordinarily was inactive due perhaps to presence of an inhibitor which was destroyed by acetone drying or long aeration.

Although we have been unable to confirm synthesis of pantothenic acid in aerated cultures of the mutant strain, it is quite possible that unrecognized variables in the cultural conditions are responsible for this difference in result. Failure of growing cultures of the organism to synthesize pantothenate is, of course, consistent either with absence of the coupling enzyme or with inactivity of this enzyme during growth due to presence of an inhibitor.

Summary. 1. Synthesis of pantothenic acid and related compounds by wild type *Neurospora crassa* and a *pantothenicless* mutant of this organism, 5531A, was compared by growing these organisms for 4-8 days and determining the substances produced in the culture filtrates by microbiological and chromatographic methods. Culture filtrates of wild type *Neurospora* at 4 days contained principally coenzyme A; on longer incubation the coenzyme A decreased while the pantothenic acid and pantetheine (LBF) in the medium increased. Little or no pantoic acid

was present. Culture filtrates of the *pantothenicless* mutant contained substantial amounts of pantoic acid which arose by synthesis; no synthesis of pantothenic acid or coenzyme A was observed when the culture was grown with minimal amounts of pantothenic acid. When larger amounts of pantothenic acid were added, small amounts of coenzyme A were detected in the culture filtrate. 2. Of the compounds so far implicated as intermediates in synthesis of coenzyme A, only pantothenic acid supports growth of this *pantothenicless* mutant.

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Adenovirus Neutralizing Antibody Determination by Colorimetric Assay.* (22937)

PAUL B. JOHNSTON,[†] J. THOMAS GRAYSTON,[‡] CLAYTON G. LOOSLI

Department of Medicine, University of Chicago School of Medicine, Chicago, Ill.

The newly recognized group of adenoviruses(1) isolated primarily from the respiratory tract(2,3) has been shown to be made up of at least 14 types separated on the basis of neutralizing antibody(4). To facilitate our study of this group of viruses a more simplified procedure for determining adenovirus neutralizing antibody in HeLa cell tissue culture was developed(5). In this test serum, virus antigen, and HeLa cell suspension are added to test tube at one time. If the virus antigen is neutralized by the serum, normal growth of HeLa cells occurs. If the serum does not contain sufficient antibody cytopathogenesis of the HeLa cells takes place and can be seen after 24 to 48 hours incubation. In our experience this procedure has proven somewhat more accurate, considerably easier and more rapid to perform than the customary method of inoculating the serum virus combination into previously prepared tissue cultures.

The present report describes a still more simplified method by colorimetric assay for determining adenovirus antibody. This procedure depends on the previously reported phenomenon that growth of adenovirus in HeLa cell cultures causes lowering of pH of medium below that of uninfected cultures and that specific immune serum prevents this pH effect(6). By quantitating the pH lowering effect of adenovirus growth in HeLa cells in relation to serum dilution the amount of specific antibody can be determined. The pH lowering effect occurs in 3 days and the serum neutralizing end points are determined by gross inspection of color of phenol red indicator. For further ease of performance the

entire reaction has been carried out in cups of a disposable plastic panel.

Materials and methods. HeLa cells. The HeLa cell line employed was obtained from Dr. Jerome T. Syverton in 1953 and has been maintained in this laboratory. This line now grows readily in culture medium to which either human or horse serum has been added. Stock cultures of HeLa cells were grown in Eagle's basal medium(7) to which 10% human serum was added. Recently it has been found that the S3 clone HeLa cell line developed by Puck and associates(8) may be employed in the test with equally satisfactory results. *Adenovirus prototypes* 1 through 10 were obtained from Dr. R. J. Huebner. Type 4 (RI-67) was supplied by Dr. M. R. Hilleman. Stock antigens were prepared in HeLa cell cultures grown in 32 ounce prescription bottles. Type specific rabbit antisera were prepared by inoculating intravenously rabbits with 10 ml of a given type of adenovirus followed by intraperitoneal inoculation of 10 ml of the same antigen one week later. The rabbits were then bled one month after the first injection. Paired serum samples from persons suffering acute respiratory disease and from whose throat washing an adenovirus was isolated were employed in the evaluation of the test. These were the same serum samples previously used in the comparative study of "conventional" and "improved" neutralization technics(5,9). Each serum sample was heat inactivated (56°C for 30 minutes) and twofold dilutions from 1:4 to 1:32 and then fourfold dilutions to 1:2048 were prepared. *Medium.* For all components of the test and for all dilutions Eagle's basal medium with 10% horse serum[§] was employed. Hanks' balanced salt solution(10) with .035% sodium bicarbonate was substituted for Earle's salt solution called for in Eagle's formula(7).

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[†] Seymour Coman Fellow.

[‡] Formerly Fellow of Natl. Foundation for Infantile Paralysis.

[§] Obtained from Microbiological Associates, Washington, D.C.

Phenol red^{||} pH indicator was used at concentration of 0.002% in Hanks' solution. After adding the rest of the constituents of the basal medium and the horse serum, the final indicator concentration was 0.0016%. To all media 100 units of penicillin, 20 units of mycostatin and 0.1 mg of streptomycin per ml were added. Plastic panels[¶] used have been described(11). They were molded from white opaque styrene sheets and contained 96 cups in 12 rows of 8 each. Each cup has maximum capacity of 2.2 ml. Mineral seal oil^{**} was used as a seal for the cultures. It is a light oil with viscosity of 43 Saybolt seconds Universal at 100°F and with flash point of 260°F. The oil was used as received since it was always sterile when cultured on blood agar. Each of 4 separate lots have proven to be satisfactory.

Procedure. Before each test the plastic panels are rinsed in distilled water, dried in air and irradiated for about 15 minutes with ultraviolet light. 0.5 ml of mineral seal oil is first added to each cup with 2 ml Cornwall pipette. The serum, virus antigen and HeLa cells then are added consecutively through the oil. 0.1 ml of appropriately diluted serum is added to each cup with a mouth pipette. One cup per serum dilution is used.^{††} The adenovirus antigen (4 units, see below) in 0.2 ml amounts is next added to each cup with 1 ml Cornwall pipette. The serum-virus mixture is allowed to stand at room temperature for at least 30 minutes before HeLa cells are added. 0.5 ml of newly harvested HeLa cell suspension (trypsin method) containing approximately 100,000 cells, by hemocytometer

chamber counts, is then added rapidly to each cup with a Brewer automatic pipette. After addition of all materials each cup then contains a total volume of 1.3 ml. The following controls are included in each run: (a) Cell controls: HeLa cells alone; (b) Serum controls: HeLa cells plus serum (0.1 ml of the lowest dilution of each serum tested along with 100,000 HeLa cells); (3) Virus controls: HeLa cells plus virus (0.2 ml of each virus antigen with 100,000 HeLa cells). For each antigen type employed a test against its type specific rabbit antiserum also is included. The plastic panels containing above ingredients are then incubated at 36°C and observed periodically for pH color change.

The final reading of a test is made when the medium of virus controls becomes yellow in color. This is usually after 2½ to 3 days of incubation. An occasional test had a sharper end point in 3½ days. The end point was usually still clear after 4 and 5 days incubation. During incubation the purple color of the medium of HeLa cell control (pH 7.8-7.6) changes only to red orange to orange (pH 7.5-7.3) while that of virus controls become yellow to lemon yellow (pH 7.0-6.8). The color of medium in test cups containing serum, virus antigen and cells varies from red to orange to yellow as the end point of serum dilution is reached. To give a numerical value to color changes, the pH of cups is determined by comparison with a set of phenol red standards with 0.2 pH intervals ranging from 6.8 to 7.8. In actual practice the serum neutralizing end points can be determined rapidly by direct inspection of color in the cups. In cups containing serum, virus and cells, a full orange color indicates neutralization while a full yellow color signifies no neutralization. An intermediate yellow-orange color seen at end point in a series of 2-fold dilutions of serum is considered to show neutralization if the pH in the cup containing this dilution is about 0.2 unit above pH of the virus control. The antibody titer in a given serum is expressed as the reciprocal of the highest serum dilution showing evidence of neutralization. An example of colorimetric neutralization test is shown in Fig. 1.

^{||} Phenol red stock solution was prepared from 0.4% aqueous suspension, dissolved by careful neutralization (to avoid excess) with sodium hydroxide.

[¶] Obtained from Linbro Chemical Co., New Haven, Conn.

^{**}Trade name of Standard Oil Co. (Indiana) who kindly supplied the mineral oils tested. Other heavier mineral oils with higher viscosities were less satisfactory since their use slowed the lowering of pH of the virus culture system.

^{††} It was found that plates containing the oil and serum could stand for at least one week in icebox at 4°C before the virus antigen and HeLa cells are added without altering results of the test.

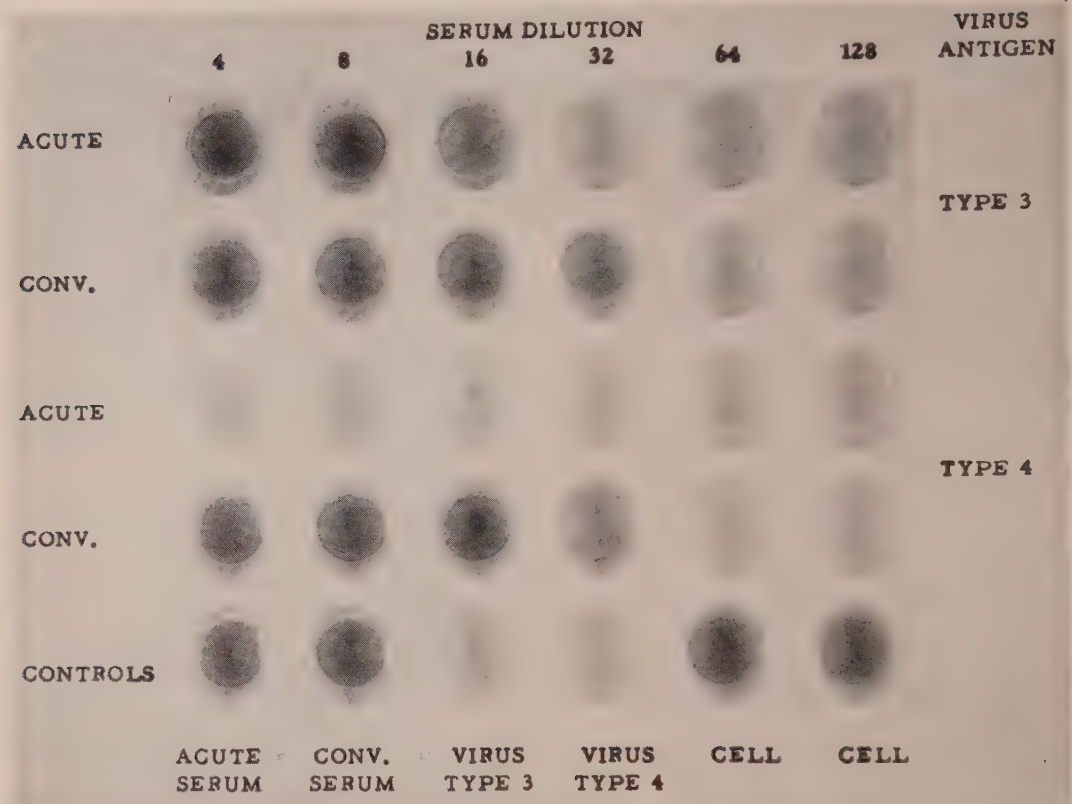


FIG. 1. Photograph of plastic panel showing final reading (3 days) of adenovirus-HeLa cell colorimetric neutralization test with phenol red indicator. (Conv. = convalescent serum.)

The paired serums were from an individual who had a febrile respiratory disease and from whom a type 4 adenovirus had been isolated from the throat washing in the acute phase of illness. The convalescent blood was taken approximately 3 weeks after onset of illness. In the test shown in Fig. 1 serum antibodies for adenovirus antigens, types 3 and 4, are determined. The photograph of the plastic panel with the various ingredients of the test was taken after 3 days of incubation. The dark cups, such as cell and serum controls, have an orange red color and a pH of approximately 7.4 while the light cups such as virus controls have a lemon yellow color and a pH of 6.8. An intermediate color at end point of the 2-fold serum dilutions can be seen particularly at the 1:32 dilution of the convalescent serum against type 4 antigen. In the test shown in Fig. 1 the neutralizing titers in acute and convalescent serum

against type 3 antigen were read respectively as 16 and 32 while those for type 4 antigen were less than 4 and 32.

Adenovirus antigen titration. Twofold dilutions to 2048 of stock adenovirus types 1 through 10 are placed in plastic panel cups as described for virus controls and incubated for 3 days. The highest viral dilution showing a definite color change, compared to that of the cell control during this period of time, is considered the virus titer (one unit). A fourfold more concentrated suspension (4 units) is then employed in the neutralization test. This amount corresponds closely to that used in the previously reported simplified test tube microscopic procedure(5). In Table I are shown examples of titrations of the 10 adenoviruses employed. The end points of titrations are underscored and varied from 1:8 for type 8 to 1:256 for types 1, 2, 3 and 6. It can be noted that the end

TABLE I. Examples of Adenovirus Titrations* of Each of 10 Types in Plastic Cups Read by Inspection of Color Change of Phenol Red pH Indicator. The virus titer underscored is considered one unit.

Phenol red indicator		Adenovirus type									
pH	Color†	1	2	3	4	5	6	7	8	9	10
6.8	lemon	16	32	32	8	16	32	8	2	8	16
	yellow	32	64	64			64				
6.9		64	128		16	32	128	16		16	32
7.0	yellow	128		128		64	256		4	32	
7.1		256	256	256	32		512	32	8		64
7.2	yel-or					128	CC		16		
7.3	orange	512	512	512	64	256		64	32	64	128
				CC		CC			CC	CC	CC
7.4	red-or	CC	CC		CC			CC			

* Reciprocal of stock virus dilution.
CC = Cell control.

† yel-or = yellow-orange; red-or = red-orange.

points represent color change brought about by lowering of pH approximately 0.2 unit compared to pH of the corresponding cell control (CC). After 3 full days incubation the dilution of virus 4-fold more concentrated than the end point dilution produced in each case a yellow color. The development of a full yellow color never occurred in less than 2 days even with undiluted virus.

Results. Rabbit type specific antisera had been prepared against adenovirus types 1 through 10. Each of these sera had been previously titered with the test tube microscopic neutralization technic. Since these sera were included as positive controls of each antigen in each test several repeat runs

utilizing the colorimetric assay method in plastic cups are available to study repeatability and to compare with results obtained by the prior methods. Table II summarizes the results obtained with these rabbit sera. As many as 21 repeat runs have been performed with type 3 antiserum. Repeatability of end points was close with each antigen. Only one determination (with type 7) fell out of a 4-fold range. The average titer obtained with each type was that reported with the previous method(5) except in the case of type 5 which was 2 to 4 fold higher. Results with 2 different type 9 rabbit antiserum are shown in Table II because the serum of rabbit "P" had been previously reported(5),

TABLE II. Studies of Repeatability of Neutralization Titers in Type Specific Rabbit Antisera, Types 1 through 10, with Colorimetric Method and Comparison of These Titers with Those Previously Obtained with the Test Tube Microscopic Method.*

Adenovirus antigen type	Microscopic method, avg titer	Colorimetric method						
		No. of tests with indicated neutralizing titer						
		32†	64	128	256	512	1024	2048
1	128		1	3	2			
2	512				1	1	2	
3	512					16	3	2
4	512					6	1	2
5	256					2	2	
6	512					2	3	
7	512			1		7	3	1
8	128			2	1			
9 "P"	64	1						
9 "R"	512					2	1	
10	128		2	1	1			

* Reference (5).

† Titer expressed as reciprocal of serum dilution.

while the higher titered serum of rabbit "R" has been employed as positive control in recent tests.

Each of type 1 to 10 rabbit antisera has been titered against types 1 to 10 virus to prove type specificity of neutralizations by the color test. The same high specificity resulted as reported with these sera in the microscopic test(5). Newly isolated types 3, 4 and 7 adenoviruses have been successfully typed with this colorimetric method by using undiluted virus and 1:30 dilution of these rabbit antisera.

Human serum. The neutralization titers present in paired serum from 53 persons suffering acute respiratory illness and from whom adenovirus had been isolated had been determined by the tube microscopic method against adenovirus antigen types 1 through 10(5,9). Sera were available on 47 of these persons for comparative testing in the colorimetric assay method. Table III presents a comparison of results between the two methods with both acute and convalescent serum titers against all 10 antigens tabulated together. A total of 812 comparisons were made with almost half showing no titer with either test. There was in general close repetition of results. In 10 tests the titer obtained with the colorimetric method was 8-fold higher and one test 8-fold less. Once the colorimetric test showed no titer against type 6 antigen in a serum previously read as having a titer of 32. Except for these 12 tests the other 800 repeat tests had a titer within fourfold of the tube microscopic determination.

Since 4-fold serum dilutions had been employed above 1:8 in the tube microscopic technic and 4-fold dilutions were used in the colorimetric assay method above 1:32 these 4-fold variations frequently meant the repeat was within one tube dilution. A definite tendency can be seen in Table III for the colorimetric assay method to give slightly higher titer result. There were 44 repeat tests that were 2- or 4-fold lower with the colorimetric method but there were 200 repeats that were 2- or 4-fold higher.

The changes in titer that were observed did on occasion result in 4-fold rises being present

TABLE III. Comparison of Neutralizing Titers with Adenovirus Antigens Types 1 through 10 Using 2 Technics with the Same Paired Human Serum Samples from Persons Suffering Acute Respiratory Illness and Having an Adenovirus Isolated from Their Throat.

Titer with colorimetric method	Neutralizing titer with test tube microscopic observation method*										
	0	2	4	8	16	32	64	128	256	512	
0	386	29	16	6	1						
4	31	16	10	1	1	1					
8	5	10	13	29	5	4					
16			2	6	2	2	1				
32			4	31	24	41	5	3			
128						38	13	24			
512							1	11	2	10	

* Reference (5,9).

between acute and convalescent sera in one test procedure but not the other. There were 5 rises observed with the tube microscopic technic alone and 15 only with the colorimetric method. None of these 4-fold rises seen in only one test were with the adenovirus type isolated from the patient.

Discussion. The use of a colorimetric assay method has allowed large scale determination of poliomyelitis neutralizing antibodies(12). With the numerous adenovirus types a simple technic is essential to determination of neutralizing antibody in large epidemiological studies. The colorimetric assay method in plastic panel cups has allowed many serum samples to be tested simultaneously for neutralizing antibody with 10 different adenovirus antigens. In addition to paired serum in association with infection neutralizing antibody response to adenovirus vaccine has been successfully studied with this colorimetric technic(13). The colorimetric procedure has also provided rapid accurate typing of newly isolated adenoviruses.

The color reaction employed in this test is the opposite of that used in poliomyelitis neutralization test(11,14-17) where inhibition of cellular metabolism by the poliovirus results in a higher pH than in uninfected or neutralized virus cultures. The pH lowering effect associated with adenovirus growth in HeLa cell tissue culture allows more rapid pH indicator changes than metabolic inhibi-

tion. This more rapid (3 day) determination of neutralizing effect is particularly valuable with the adenovirus group due to their tendency to readily "break-through" the neutralizing effect of antisera.

Summary. A colorimetric assay method for measuring neutralizing antibodies against adenovirus in HeLa cell tissue culture in plastic panel cups is described. Advantage is taken of the fact that adenovirus growth in HeLa cell cultures lowers the pH of the medium below that of uninfected cultures. The reaction is standardized so that in 3 days the phenol red indicator has turned yellow by unneutralized virus while the indicator has turned only to red orange by the metabolism of the HeLa cells when the virus antigen is neutralized. It has been shown that this colorimetric test gives titers in paired sera from human adenovirus infections with antigen types 1 through 10 which are similar or slightly higher than those obtained with a test tube method depending for reading on microscopic observation of cytopathogenesis. This method has also been found suitable for typing adenoviruses.

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Dibenzamine Blockade of Epinephrine and Glucagon Hyperkalemias.* (22938)

SYDNEY ELLIS, SIBYL B. BECKETT† AND JOSEPH H. BOUTWELL‡

Departments of Pharmacology and Physiological Chemistry, Temple University School of Medicine, Philadelphia, Pa.

Although the liver is the major source(2,3)

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† Present address: Dept. of Physiology and Pharmacology, Univ. of Pennsylvania Graduate School of Medicine, Philadelphia, Pa.

‡ Present address: Chemistry Laboratories, National Naval Hospital, Bethesda, Md.

of increased plasma potassium which follows intravenous injection of epinephrine(1), the mechanism by which hepatic release of potassium occurs is still unknown(4). The hyperkalemic response to epinephrine has not been correlated definitively with either vasopressor or hyperglycemic response to epinephrine (2,5,6,7). Nonetheless, some findings suggest an association between potassium and

glucose release from the liver. Thus, Verzar and Somogyi(8) observed that epinephrine did not elevate plasma potassium or glucose in adrenalectomized cats which showed marked evidence of adrenal insufficiency, but when adrenalectomized cats were maintained on desoxycorticosterone, epinephrine produced essentially normal elevations in plasma potassium and glucose. Recently, it was found that glucagon, which appears to mimic the glycogenolytic action of epinephrine on the liver but to act only on that organ, produces hyperkalemia as well as hyperglycemia (9). Some reports on ability of adrenergic blocking agents to antagonized hyperkalemic response to epinephrine suggest an independence of the hyperglycemic and hyperkalemic responses. The fact that ergot derivatives antagonize the hyperkalemic(1,3,10) is in keeping with their antagonism toward the hyperglycemic and most other responses to epinephrine. This action may indicate that these alkaloids prevent access of epinephrine to the reactive sites ("receptors") in the liver cell. On the other hand, the ability of dibenamine to blockade the hyperkalemic response to epinephrine in cats(11) and in dogs (10) is unexpected in view of the extremely weak antagonism of dibenamine against epinephrine hyperglycemia in cats(12). From these reports one could not safely conclude that dibenamine selectively blockaded potassium release from the liver without blockading glucose release since none of the investigators had studied both responses.

In the present report we have attempted to supplement previous work by determining the epinephrine-induced changes in plasma potassium and glucose in the cat before and after administration of dibenamine. Since we confirmed the findings of other investigators, that dibenamine has little effect on epinephrine hyperglycemia but has remarkable ability to prevent epinephrine hyperkalemia, we have investigated the ability of dibenamine to blockade glucagon hyperkalemia. The latter study was important for determining selectivity and mode of action of dibenamine since the glycogenolytic effect of glucagon appears identical with that of epine-

phrine(13) except for the fact that the cell "receptors" for glucagon and epinephrine differ(14,15).

Methods. The experiments were performed upon cats anesthetized with Dial. The methods used were the same as those reported previously(9), except for analysis for plasma potassium which was done on Baird Associates Flame Photometer with an internal lithium standard. Epinephrine bitartrate (10 $\mu\text{g/kg}$) and glucagon (50 $\mu\text{g/kg}$) in volumes of approximately 0.1 ml were injected rapidly into the femoral vein. Dibenamine dissolved in 50% ethyl alcohol and diluted appropriately with 0.9% sodium chloride solution so that a total dose of 15 mg/kg in a volume of about 15 ml was administered in 15 minutes by continuous infusion. One hour was allowed after completion of the infusion of dibenamine before testing of either glucagon or epinephrine. Arterial blood samples were taken before and 1, 10, and 30 minutes after administration of the hyperkalemic agent. Since maximal increases in plasma potassium occur 1 minute after and maximal increases in plasma glucose occur 10 minutes after administration of either epinephrine or glucagon, only these two values will be included in this report.

Results. Observations summarized in Fig. 1 indicate that dibenamine suppresses selectively the hyperkalemic responses to epinephrine and glucagon, but that dibenamine does not markedly depress the hyperglycemic responses to epinephrine or glucagon. The results with epinephrine confirm in the same animals results previously reported on independent studies of the blockade by dibenamine of epinephrine hyperkalemia in the cat (11) and in the dog(10) and the very small effect of dibenamine on epinephrine hyperglycemia in the cat(12).

The effect of dibenamine on hepatic release of potassium cannot be the result of a blockade of epinephrine receptors in the liver. Dibenamine did not influence the ability of epinephrine to activate the glycogenolytic system of the liver. For this glycogenolytic effect epinephrine must first combine with the receptors but this step is circumvented in

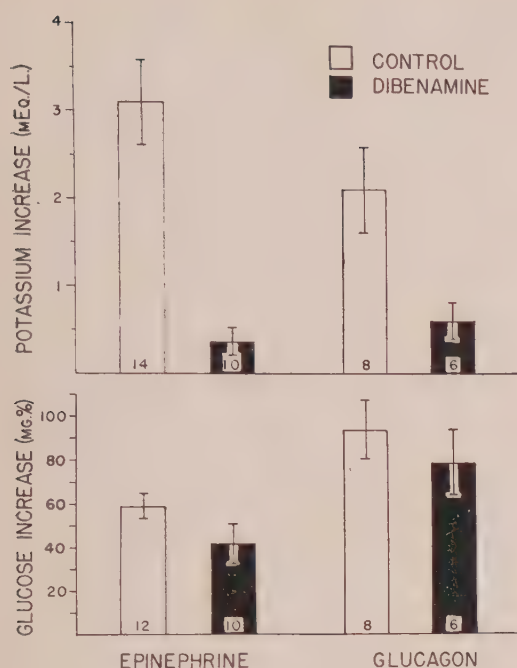


FIG. 1. Effects of epinephrine and glucagon on plasma potassium (1 min after injection) and on plasma glucose (10 min after injection) in control and in dibenamine-treated cats. Heights of columns indicate averages of the number of experiments designated at bottom of each column and vertical bars show standard error of mean.

the action of glucagon(15). Thus, it is possible to blockade the glycogenolytic effect of glucagon without interfering with the glycogenolytic effect of epinephrine(14), and *vice versa*(15).

Blockade of the hyperkalemic response to glucagon by dibenamine, as in the experiments with epinephrine, was not accompanied by a marked reduction in hyperglycemic response to glucagon. This result suggests that the blockade of the hyperkalemic responses to epinephrine and to glucagon is not a specific adrenergic blocking action of dibenamine. It would appear more probable that this action of dibenamine is some new action of dibenamine more specifically directed toward potassium metabolism. Of interest in this connection is the observation by Huggins *et al.*(16) that dibenamine increased the volume of distribution of intravenously administered potassium. Our analyses of the

potassium content of liver water indicate that in cats and rats dibenamine increases liver potassium, so that the dibenamine action cannot be attributed to depletion of hepatic potassium.

Summary. In cats the hyperkalemic response to epinephrine or to glucagon was blocked by dibenamine, but dibenamine had little effect on the hyperglycemic response to epinephrine or to glucagon. The results suggest a selective action of dibenamine on potassium metabolism in the liver.

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Influence of Inositol on Peripheral Vascular Responses to Cold Injury. (22939)

BERNARD J. SULLIVAN AND PATRICIA M. FLYNN. (Introduced by W. C. Moloney)

Biology Department, Boston College, Chestnut Hill, Mass.

In the study of the effects of cold injury on living tissues a great deal of attention has been centered on vascular disturbances. Loss of plasma and packing of blood cells, as a result of cold injury, eventually lead to intravascular agglutinative thrombi and vascular failure (Greene, 1). It has been indicated that loss of plasma and consequent stasis is due to alteration of permeability of capillary walls (Fuhrman and Crismon, 2). Furthermore, vascular responses in areas remote from the injury are caused by toxic substance originating in the damaged tissues (Lewis, 3). Additional evidence that vascular constrictions and capillary damage are produced by a blood-borne substance has been presented by Sullivan and Kelley (4). Numerous attempts have been made to offset the effects of cold injury. Rapid rewarming of damaged tissues and anticoagulant therapy have been widely used but with controversial results. The present investigation involves the use of inositol, which combines anticoagulant activity with the property of protecting against capillary fragility. The antithromboplastic action of inositol was demonstrated by Kay and Balla (5). The fact that it decreases capillary fragility was reported by Belli, *et al.* (6). Both of these factors were taken into consideration in conducting this investigation. Observations were made to ascertain whether inositol can inhibit formation of agglutinative thrombi, reduce plasma loss by its capillary-protective action, and consequently, prevent stasis in peripheral vascular areas remote from cold injured tissues.

Materials and methods. Mature hamsters (*Mesocricetus auratus*) weighing 85 g to 120 g were used. Anaesthetization was achieved by intraperitoneal injection of Nembutal sodium (Abbot, 50 mg/cc). An initial dosage of 0.2 cc was administered, with additional dosages of 0.15 cc if required. Anaesthetized animals were placed on plexiglass microscope

stage and the cheek pouch was everted and prepared according to the technic of Fulton, *et al.* (7). A small, incised flap of the pouch was spread over a glass block and pinned to paraffin moat exposing thin layer of tissue for microscopic examination. During preparation of the pouch and in the ensuing period of observation, the exposed tissues were continuously irrigated with physiological saline buffered to pH 7.4, and maintained at constant temperature of 37.5°C. The vascular components of the pouch were examined under magnifications of 100x to 200x. Observations were made on 4 series of animals; 1) normal, untreated controls, 2) cold injury controls, 3) those subjected only to inositol injections, and 4) those receiving inositol 10 minutes prior to induction of cold injury. All animals received similar doses of Nembutal and were under observation for 2.5 to 3 hours. Cold injury was produced by wrapping a hind limb to the knee joint in an aluminum foil boot containing pulverized CO₂ ice. It was held in place for one minute. Inositol was administered by intraperitoneal injection of 1 mg/100 g body weight. Inositol was dissolved in buffered saline and injected at 37.5°C.

Results. No spontaneous variation in diameter of the vessels was observed in untreated controls under the experimental conditions. Rate of flow was constant, although slightly more rapid on the arterial side of capillaries than on the venous side. A marked laminar flow was evident in arteries, but a more uniform flow was observed in veins. The direction of flow was subject to occasional fluctuations.

In cold injury controls the typical three-phase response (Sullivan, 6) was apparent. Application of dry ice to the hind limb produced (1) an immediate generalized increase in rate of blood flow. This was followed, after removal of the cold source, by (2) a

gradual decline in rate of flow. The final phase (3) observed 10-20 minutes after onset of thaw, was characterized by marked arteriolar constrictions, coatings of blood cells on the venous endothelium, formation of thrombi, retardation of venous flow, intravascular packing of blood cells, and stasis.

In animals subjected only to inositol injections there were no deviations from the normal vascular pattern and reactions. However, in the preparation of the cheek pouch these animals revealed a marked tendency to profuse hemorrhage when even a small vessel was damaged.

In animals treated with inositol before induction of cold injury the initial phase of rapid flow and the secondary gradual decline were apparent. There was no deviation from cold injury controls in either degree of change or time factor. The constriction phase appeared in the usual time after onset of thaw (10-20 minutes). Sluggish flow was apparent in veins but this condition was not as prominent as in cold injury controls. A slight endothelial coating of cellular elements was observed in some of the smaller venules but this was not as widespread nor as severe as in the controls. The number of functional capillaries was somewhat reduced but in the open vascular beds the rate of blood flow appeared normal. In general, there was a definite alleviation of the conditions which normally follow cold injury, and no case of complete stasis was observed in the inositol treated animals.

Discussion. It has been pointed out that peripheral vascular responses to cold injury are due primarily to loss of plasma through impaired capillary walls(2) and the formation of agglutinative thrombi(8). These conditions lead to intravascular packing of blood cells, occlusion of vessels, and ultimately, complete stasis. Local cold injury suffered by the tissues is dependent specifically upon the extent of these conditions and the resultant tissue anoxia(Shumacker,9). From a

theoretical viewpoint it would seem that any agent which prevents or ameliorates these vascular disturbances would promote and maintain a more adequate blood flow, reduce anoxia, and decrease tissue damage.

The results of the present investigation indicate that inositol has the requisite properties of decreasing capillary impairment, inhibiting formation of thrombi, and preventing post-constriction stasis. Consequently, an adequate blood volume is maintained and the usual post-thaw packing of vessels with stranded cells does not occur. Presumably, the improved peripheral blood flow increases the supply of oxygen to the injured tissues, and despite the increased metabolic rate, should result in less necrosis.

Summary. Control animals receiving inositol injections showed no deviation from the normal vascular pattern or reactions. However, the concentration of inositol administered (1 mg/100 g of body weight) markedly increased the tendency to hemorrhage. When inositol was injected into animals prior to induction of cold injury the initial responses of frostbite controls occurred without deviation. However, the constriction phase was ameliorated, thrombi formation was inhibited, and complete stasis was eliminated.

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Atrophy of Adrenal Gland Following Thiouracil and Vit. B₁₂.^{*} (22940)M. X. ZARROW, L. M. HORGER[†] AND J. L. MCCARTHY*Department of Biological Sciences, Purdue University, Lafayette, Ind.*

Although adrenal atrophy following thiouracil treatment has been demonstrated by numerous investigators(1-7), the evidence has not been unequivocal due to body weight loss that occurs concomitantly with administration of this drug. Thus, it has been also suggested that involution of the adrenal gland is merely the result of generalized inhibition of body growth(8-10). Recently, evidence has been presented to indicate that vit. B₁₂ can prevent growth inhibiting action of thiouracil (11). Consequently, the present experiment was designed to determine whether adrenal involution could be obtained in the absence of marked depression in body growth through simultaneous administration of thiouracil and vit. B₁₂.

Material and methods. Male rats (Sprague Dawley strain) were used. All animals were placed on treatment when they reached approximately 100 g in body wt. The thiouracil was prepared as a 0.1% solution and administered in drinking water. Vit. B₁₂[‡] was prepared in saline solution and injected intraperitoneally. The animals were divided into 7 groups and treated for 12 weeks to insure definite atrophy of the adrenal cortex(3). Groups A and B were kept as controls; group A receiving no treatment and group B receiving 0.1 ml of saline intraperitoneally daily. Groups C, F and G received 0.1% thiouracil in drinking water and in addition F and G received 10 and 20 μ g of B₁₂ respectively. Groups D and E received only 10 and 20 μ g of B₁₂ respectively. In all instances the vitamin was given daily via intraperitoneal route. Body weights were obtained weekly and the animals were killed after 12 weeks of treatments. Thyroid and adrenal weights were

obtained at time of postmortem and weighed to nearest 0.1 mg.

Results. The data in Table I indicate that vit. B₁₂ prevents growth inhibiting action of thiouracil. Normal, control rats injected with 0.1 ml saline showed final average body weight of 223 g; thiouracil treated rats a body weight of 147 g and rats given thiouracil and 10 or 20 μ g of B₁₂, a body weight of 193 and 205 g respectively. The difference between body weights of thiouracil-treated rats and saline controls is significant whereas no significance is seen between the controls and groups receiving thiouracil and 20 μ g of B₁₂ simultaneously. Treatment with 10 μ g of B₁₂ partially prevented body weight depression due to thiouracil while 20 μ g of B₁₂ gave complete protection.

Thiouracil induced the expected hypertrophy and hyperplasia of the thyroid but no significant difference in weight of thyroid can be noted between groups receiving thiouracil alone and thiouracil plus B₁₂. Indication of a possible decrease in size of thyroid gland of rats treated with B₁₂ may be seen, if comparison is made of weight of the thyroid expressed as per cent of body weight. However, a significant decrease in weight of the thyroid gland of the thiouracil-treated rat is seen only with the group receiving 10 μ g of B₁₂.

Adrenal glands of untreated controls and saline controls weighed 64.7 and 53.9 mg respectively. This is a difference of approximately 20% and is significant although we can suggest no reason, since one might have expected the increase in saline controls, if at all. Adrenal glands of rats treated with thiouracil weighed 19.3 mg or 12.9 mg% body weight. Treatment with 10 and 20 μ g of B₁₂ alone resulted in adrenal glands of 24.8 and 25.4 mg% respectively, indicating no effect as compared with saline controls. The combined treatment with thiouracil and vit. B₁₂ at 10 and 20 μ g gave adrenal weights of 28 ± 7 and 33 ± 8.5 mg (Table I). Statistical

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[†] Fellow of National Science Foundation 1955-1956. Present address: Sherman Laboratories, Detroit, Mich.

[‡] Vit. B₁₂ was obtained through the courtesy of Dr. Michaud, Merck & Co., Rahway, N. J.

TABLE I. Effect of Thiouracil and Vit. B₁₂ on Body Weight, Thyroid and Adrenal Cortex of the Rat.

Treatment	Group	No. of rats	Body wt, g	Thyroid		Adrenal cortex	
				mg	mg % body wt	mg	mg % body wt
Controls	A	8	229.4 ± 21.2*	14.8 ± 1.87	6.5 ± .58	64.7 ± 10.04	28.2 ± 2.38
Saline	B	8	223.1 ± 11.1	13.6 ± 1.04	6.1 ± .58	53.9 ± 4.96	24.1 ± 1.59
Thiouracil	C	11	147.1 ± 32.3	51.5 ± 4.06	35.1 ± 7.34	19.3 ± 7.85	12.9 ± 2.48
10 µg B ₁₂	D	10	226.4 ± 18.2	12.7 ± 2.49	5.6 ± .98	56.0 ± 4.49	24.8 ± 2.69
20 " "	E	7	253.8 ± 18.2	12.2 ± 2.49	4.8 ± .99	64.4 ± 4.20	25.4 ± 1.36
Thiouracil + 10 µg B ₁₂	F	10	193.2 ± 17.5	49.4 ± 7.54	25.6 ± 3.32	28.0 ± 7.17	14.4 ± 2.75
Thiouracil + 20 µg B ₁₂	G	10	205.4 ± 26.7	59.3 ± 8.80	29.8 ± 8.40	33.0 ± 8.45	15.8 ± 2.55

* Stand. dev.

analysis revealed no significant difference between absolute weight of adrenal gland of rats treated with thiouracil or thiouracil and 10 µg B₁₂. A significant difference was noted at 20 µg B₁₂ dosage but it should be noted that the adrenals still showed marked atrophy (approximately 40%) when compared with controls. A further comparison of adrenal gland weight when expressed as a function of body weight, revealed a lack of any statistically significant difference between the adrenal gland of rats treated with thiouracil or a combination of thiouracil and vit. B₁₂.

These data confirm the report by Meites(8) in that vit. B₁₂ prevents body growth inhibiting action of thiouracil. In spite of this action, however, the vitamin was unable to prevent adrenal atrophy induced by thiouracil. Since this experiment was able to separate the action of thiouracil on body growth and adrenal gland, it can now be stated conclusively that thiouracil specifically inhibits the adrenal gland. The mechanism whereby this is done can not be stated at this time. Money (12) stated that the mechanism of adrenal involution following thyroidectomy or thiouracil treatment remains obscure. While we can now eliminate the possibility of a non-specific, generalized effect due to failure of body growth, the following possibilities await further clarification: 1) a possible shift in pituitary production from ACTH to TSH (13,14); 2) an independent decrease in ACTH elaboration; 3) a critical need for thyroxine in the normal growth of the adrenal gland(8); 4) a decreased utilization of the adrenocortical hormone(15); or 5) a direct

action of thiouracil on the adrenal gland. Thus far, the validity of the pituitary shift as a possible explanation has been questioned by several investigators(15,16).

Conclusions. Vit. B₁₂ prevented the growth inhibiting action of thiouracil but failed to prevent adrenal atrophy. Atrophy of the adrenal cortex was due to a specific action of the goitrogen and not a reflection of generalized inhibition of body growth.

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Toxic Effects of Fluid from the Coagulating Gland of the Guinea Pig* (22941)

JULES FREUND[†] AND GEORGE E. THOMPSON
(With the technical assistance of Anne Rothe)

*Division of Applied Immunology, Public Health Research Institute of The City of New York, Inc.
and National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.*

During a study of the antigenic properties of the genital organs of the guinea pig, it was unexpectedly observed that the fluid from the coagulating glands produced toxic symptoms and death in rabbits and in guinea pigs.

Methods. These glands are attached to the seminal vesicles (1,2); they were removed with eye forceps from guinea pigs that were exsanguinated under ether anesthesia. The fluid from the coagulating gland (FCG) was obtained, as a rule, by pressing the cut glands and clearing the fluid by centrifugation. Occasionally, the fluid was drawn from the glands *in situ* into a small syringe through a 27 gauge needle.

Results—Action of FCG in the rabbit. Following intravenous, intraperitoneal, intramuscular or subcutaneous injections, the symptoms included: miosis, salivation, cyanosis of the ears and lips, dyspnea, urination and defecation. The blood pressure was lowered and there was a variable drop in body temperature. The rabbits became prostrated and died within a few hours after injection. The first symptom was miosis which appeared in 2 to 5 minutes after injection and often lasted about one-half hour. Rabbits recovering from a sublethal dose were again susceptible. The minimal intravenous lethal dose was about 0.002 ml of FCG. Male and female rabbits showed about the same susceptibility. Pathological findings included dilation of the right heart, petechial hemorrhages on the surface of the thymus; the spleen and the liver were dark red, the large veins of the abdominal cavity were distended. Histological sections of the abdominal organs showed that the capillaries and venules, as well as the

sinuses of the liver and spleen, were dilated and contained clumped red cells. Extracts of other organs of guinea pigs, such as prostate, testis, epididymis, spleen, and liver did not produce parasympathomimetic effects.

Action in guinea pigs, cats, rats and mice. FCG from guinea pigs injected into guinea pigs intravenously caused urination, defecation, dyspnea and prostration, but no miosis was noted. Death occurred in one to 2 hours after injection. The lungs were not inflated. When 0.2 to 0.5 ml of FCG was injected subcutaneously regional clonus occurred lasting for a few minutes. Some of the guinea pigs developed rigidity of one or both hind legs several hours or one day after subcutaneous or intramuscular injection given regions distant from the hind legs. Of 3 cats injected intravenously with 1 ml of guinea pig FCG, 2 showed miosis and 3 intense salivation; one died and 2 recovered following a period of prostration. While large doses of guinea pig FCG produced systemic symptoms and death in rats, mice were not susceptible. FCG from rats, hamsters or mice failed to produce similar effects in rabbits, guinea pigs and rats. Extracts from prostates of the rabbit, bull, dog, or man produced no similar symptoms in the rabbit.

Instillation of a drop of guinea pig FCG diluted 1:10 into the eyes of rabbits caused no miosis but induced congestion of blood vessels and swelling of the conjunctiva and the nictating membranes. This condition lasted for one day.

It is of significance that strips of uterus and ileum of rabbits reacted with contraction to a 1:200 dilution of rabbit FCG in Tyrode solution.

The active agent of FCG is non-dialyzable, stable at 55° for 30' but destroyed by heating at 80° for 20'. It can be precipitated by half saturated (NH)₂SO₄. Incubation at 37°

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[†] Present address: National Institutes of Health, Bethesda 14, Maryland.

for 30' with fresh rabbit serum does not destroy the active agent. Preservatives such as formaldehyde (0.1%), phenol (0.5%) and merthiolate (1:10,000) do not affect it. It can be freeze-dried with little loss of its activity.

Summary. Fluid from the coagulating gland of the guinea pig injected intravenously or intramuscularly produced toxic effects (miosis, urination, defecation, salivation) and death in rabbits, recalling the parasympathomimetic action of certain drugs. When it was injected into guinea pigs intravenously,

it caused urination, defecation, prostration and death in about one hour. In guinea pigs, the intramuscular injection of this material caused regional clonus. Fluid from the coagulating glands of rats and mice injected into rabbits did not produce a similar effect.

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Comparative Effects of Ouabain Upon Contractile Force of Guinea Pig Diaphragm and Heart.* (22942)

ROBERT M. FAUST† AND PAUL R. SAUNDERS

Department of Pharmacology, School of Medicine, University of Southern California, Los Angeles.

The positive inotropic action of cardiac glycosides upon heart muscle has been well established in studies with isolated papillary muscles(1) and ventricle strips(2) and with intact heart preparations(3-5). The effect of these drugs upon the force of contraction of skeletal muscle is less clear, however. For example, no significant positive inotropic action was observed in a variety of skeletal muscle preparations(6-10). On the other hand, a number of investigations using different preparations or experimental conditions have revealed an increased force of contraction(6,7,9-16). Similarly, negative inotropic actions have been reported (7,9,15-17) as well as failure to observe such an effect with other preparations or conditions(6,8,9). The conflicting results obtained above are in part inexplicable. It might be noted, however, that in much of the work (with *in situ* as well as isolated preparations) the experimental conditions were not defined precisely, nor were concentration-action relationships explored

fully. In addition, direct comparisons of the action on skeletal and cardiac muscle of the same animal have not been made.

The effects of these drugs assumes an especial importance in view of recent work on their mechanism of action, in which skeletal muscle and contractile protein preparations from skeletal muscle have been used(18-21). The significance of such investigations is in large dependent upon demonstration that cardiac glycosides produce a positive inotropic action upon skeletal muscle. We have therefore investigated the problem in simple skeletal and cardiac muscle preparations of guinea pig *in vitro*, using well-defined conditions and a wide range of concentrations.

Methods. The isolated left phrenic nerve-diaphragm preparation of the guinea pig (150-200 g males) was used. Preparation of the strip from the diaphragm was carried out as described originally in the rat by Bülbiring (22), and the muscle stimulated with a Grass stimulator (Model S-4-B) with square wave shocks. The muscle was stimulated either directly (the base of the muscle wedge rested upon a platinum electrode of the same length as the muscle base) with a stimulus of 1 millisecond duration and 40 volts, or indirectly

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† Medical student fellow of National Foundation for Infantile Paralysis.

through the phrenic nerve with a stimulus of 0.1 millisecond duration and 20 volts. Stimuli of these intensities gave contractions of equivalent force through nerve and muscle. At the above voltages, the force of contraction on stimulation of the muscle was essentially constant on direct stimulation at durations of approximately 0.5-1.5 milliseconds and on indirect stimulation at 0.01-0.2 millisecond. Stimuli of longer duration resulted in an increased force of contraction, due presumably to multiple, "fused" contractions as described previously(23,24), while stimulation of the muscle directly at durations of about 0.5 millisecond or less usually produced contractions of less force, due presumably to submaximal stimulation. In some experiments, stimulation of the muscle directly was established by the addition of *d*-tubocurarine chloride (1-2 γ /ml), which abolished the response to stimulation through the nerve without affecting the response to direct stimulation. All experiments were performed with the muscle stimulated directly, unless otherwise noted. Krebs-Ringer bicarbonate medium, gassed with 5% CO₂-95% O₂, was used in most of the work(25). A few experiments were performed in a phosphate-buffered medium similar in composition to that used commonly in studies on the action of cardiac glycosides upon cat papillary muscles(1). The phosphate medium contained 0.9% NaCl, 0.042% KCl, 0.036% CaCl₂, and 1 mM sodium phosphate buffer. The medium had a final pH of 7.4, and was gassed with 100% O₂. Both media contained 0.1% glucose. All experiments were performed at 37°C, with the muscle preparation suspended in 150 ml of medium. The ouabain used was U.S.P. grade (Penick), and was dissolved in about 1.0 ml of medium and added to the bath. The contractions were recorded optically, using a semi-isometric torsion lever. The shortening of the muscle was magnified 59 x by the optical system, and a deflection of the beam of 4 mm corresponded to a developed tension of 1.0 g. The resting tension on the muscle was maintained at 2.0 g by means of a micrometer arrangement described previously(26), in order that any change in length of the muscle at this tension could be measured. The mus-

cle was stimulated at a rate of 6 per minute. The activity was recorded for a control period of 2 hours prior to the addition of the drug, during which time the force of contraction declined only slightly. The average force exerted by the muscle at the time of addition of the drug was 6 g. Some experiments were performed on isolated, electrically-stimulated strips prepared from the right ventricular wall of the guinea pig heart, in a manner similar to that described for the rat heart (26). The animal was killed by crushing the skull, and all manipulations involved in the preparation of the strip were done at room temperature. The strip was stimulated at a frequency of 100 per minute at a maintained resting tension of 0.75 g, and the contractions recorded optically with a semi-isometric optical lever system with which a force of 100 mg gave a deflection of 30 mm (amplification of shortening of muscle 62 x). The average force exerted by the muscle at the time of addition of the drug was approximately 100 mg. The experiments were performed in Krebs-Ringer bicarbonate medium at 37°C.

Results. Actions of ouabain upon diaphragm in bicarbonate medium. Fig. 1 shows typical experiments over a range of concentrations of ouabain. A positive inotropic action occurred at least briefly over a wide range of concentrations, with a maximal effect at 0.5 γ /ml. The higher concentrations depressed the force of contraction markedly after a brief period of stimulation, as can be seen from a comparison with the control preparations to which no drug was added. The magnitudes of both the positive and negative inotropic responses were the same, whether the muscle was stimulated directly or through the phrenic nerve. The relation between force of contraction and duration of stimulus at constant voltage was the same during the phases of positive and of negative inotropic action as prior to the addition of the drug, *i.e.*, the force was approximately constant at durations of 0.5-1.5 or 0.01-0.2 milliseconds when stimulated directly or indirectly, respectively. The changes noted in developed tension cannot, therefore, be explained on the basis of alterations in refractory period or threshold of the muscle. Changes in length

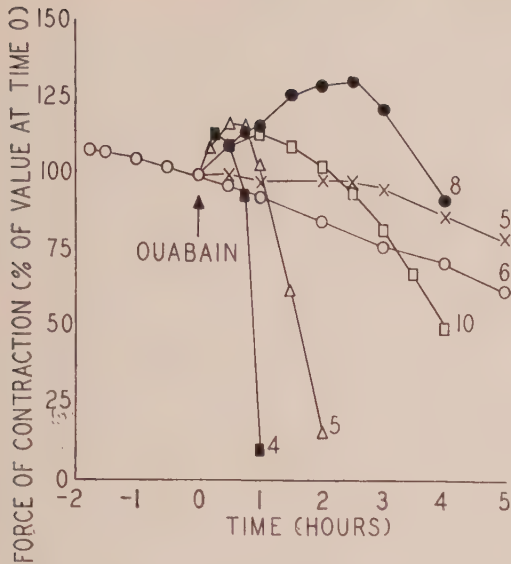


FIG. 1. Effect of ouabain upon force of contraction of diaphragm in Krebs-Ringer bicarbonate medium (muscle stimulated directly). Ouabain added at zero time above. Curves indicate average values for each concentration of ouabain (no. of experiments indicated on each curve). —○—○— controls (no drug), —×—×— 0.1 γ /ml, —□—□— 0.3 γ /ml, —●—●— 0.5 γ /ml, —△—△— 1.0 γ /ml, —■—■— 3.0 γ /ml.

of the muscle at constant resting tension during the periods of increased or decreased force were slight and unrelated in time to the changes in developed tension.

Actions of ouabain upon diaphragm in phosphate medium. In view of the fact that most investigations on the positive inotropic

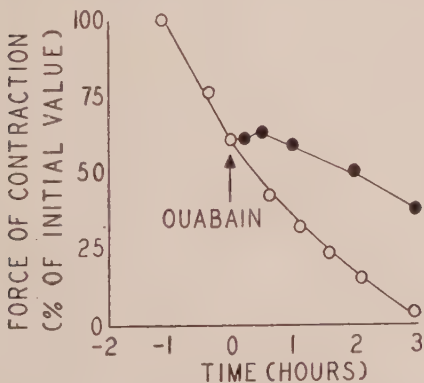


FIG. 2. Effect of ouabain upon force of contraction of diaphragm in phosphate medium (muscle stimulated directly). Ouabain (0.5 γ /ml) was added at zero time above. Curves indicate average values for 8 experiments with ouabain and 4 controls. —○—○— control (no drug), —●—●— ouabain.

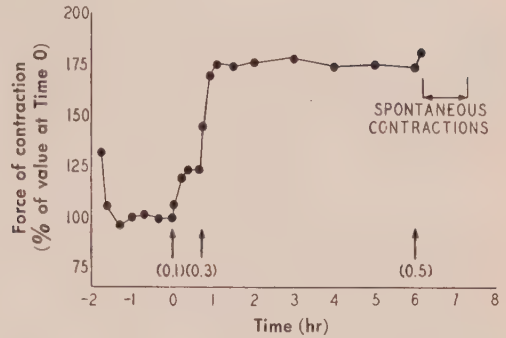


FIG. 3. Effect of ouabain upon force of contraction of ventricle strips. Ouabain added at arrows. Total concentration of ouabain in bath in γ /ml indicated in parentheses beneath arrows.

action of the cardiac glycosides on such preparations as the cat papillary muscle have been done in phosphate-buffered media(1,27), we determined the response of the diaphragm to ouabain in such a medium (see "*Methods*"). As seen in Fig. 2, the force of contraction decreased rapidly by comparison with the bicarbonate medium used above. This difference in the stability of isolated muscle preparations is well-known for the papillary muscle(27) and the rat diaphragm(28). Fig. 2 shows that some increase in force is produced by ouabain by comparison with the control, but it is of small magnitude compared with the response in bicarbonate medium (Fig. 1) or with the response of cardiac muscle in phosphate medium(1,2).

Actions of ouabain upon isolated ventricle strips in bicarbonate medium. It was of interest to compare the actions of ouabain upon the force of contraction of cardiac muscle in bicarbonate medium with those described above, and for this purpose the isolated guinea pig ventricular strip was used (see "*Methods*"). A positive inotropic effect was produced at a concentration of ouabain of 0.1 γ /ml, and a considerably greater response occurred when the concentration was increased to 0.3 γ /ml, as shown in the typical experiment in Fig. 3. Concentrations of 0.5 γ /ml resulted frequently in the appearance of automaticity, with the muscle contracting spontaneously for brief periods or for hours at a rate of 150-200/minute and independently of the frequency of the stimulator.

Such automaticity represents a toxic response to the digitalis glycosides(29). The muscle usually went into a contracture-like state following such automaticity, with a great increase in resting tension.

Thus, both a positive inotropic action and toxic effects were produced in heart and diaphragm with similar concentrations, although the magnitude of the former was greater in heart. The experimental conditions under which the drug was tested were the same with both tissues, except for the frequency of stimulation. It was not desirable to use the same frequency, as the force of contraction of diaphragm declined rapidly at high rates and on the other hand the initial developed tension of cardiac strips was very low at slow rates of stimulation. The possibility that the quantitative differences in magnitude of the positive inotropic action in diaphragm and heart might be related to activity of the muscle cannot, therefore, be determined from our results.

Actions of ouabain upon intact guinea pigs. In view of the fact that the effective concentrations of ouabain upon the diaphragm and heart were of approximately the same order of magnitude, we investigated the action of the drug in the intact animal in order to determine if skeletal muscle function was affected at doses which did not cause cardiac arrest. Doses of ouabain of approximately 0.25 mg/kg were administered intramuscularly to unanesthetized guinea pigs. Within a period of one-half hour the animals began to display signs of skeletal muscle paralysis, which reached maximum intensity in about one hour. Paralysis of the hind legs and neck muscles was marked, with the hind quarters and head resting upon the floor. The animals showed great difficulty in righting themselves when placed on their backs. At this point, electrocardiographic tracings (Lead II) were taken and compared with tracings taken prior to the administration of the drug. Prolongation of PR interval was sometimes noted, but indications of cardiac toxicity (e.g., arrhythmias) were usually not seen when the animals were tested shortly after the development of muscular paralysis. The animals were ob-

served again after completion of the electrocardiographic tracings and the paralysis was found to persist for several hours after administration of the drug. Some animals died within an hour after the appearance of the muscular paralysis, but many recovered completely.

Discussion. The present study has demonstrated that ouabain possesses a positive inotropic action upon isolated diaphragm of guinea pig. Previous studies with diaphragm of rat and mouse did not reveal such an action (6-8). Our results represent the first demonstration in an isolated mammalian skeletal muscle preparation of a positive inotropic action of a cardiac glycoside. Lendle and Oldenberg(10) observed an increased force of contraction in the masseter muscle preparation of the rat stimulated *in situ* with needle electrodes with submaximal but not with maximal stimuli; their results are in contrast with ours, which were obtained with supramaximal stimuli. They attributed the effect to an increased excitability of the muscle under the influence of the drug, but we found no change in the intensity of stimulus required to produce a maximal contraction. In view of the fact that a positive inotropic response occurred at concentrations lower than those producing paralysis, and further that the latter can occur at least in limb and neck musculature with non-fatal doses of the drug, it is possible that an increase in skeletal muscle force might occur with therapeutic doses of the drug.

No negative inotropic action of cardiac glycosides occurred in the phrenic nerve-diaphragm preparation of the rat or mouse(6-8) except in the presence of high extracellular concentrations of potassium(7), although such an action has been reported in a variety of other skeletal muscle preparations(7,9,15-17). We found that a marked skeletal muscular paralysis occurred on administration of ouabain to unanesthetized guinea pigs, prior to or in the absence of significant cardiac toxicity. Skeletal muscular paralysis has been reported to occur in the neck and leg muscles of rats(7), and respiratory paralysis occurred frequently prior to cardiac arrest on adminis-

tration of cardiac glycosides to rats(30) and guinea pigs(31-34). It is of interest that LaDue(35) reported marked skeletal muscular weakness in a patient with digitalis poisoning and a decreased work ability of dogs on a treadmill following digitalis administration. The incidence and possible clinical significance of muscular paralysis in digitalis-poisoned patients might well be explored further.

Our results imply that whatever system(s) in cardiac muscle are affected by the cardiac glycosides with resultant increased force of contraction are probably also present in skeletal muscle, and hence the latter could reasonably be used in studies on the mechanism of action of the drugs. In view of the clinical use of the drug and the smaller effect upon the diaphragm, however, it would appear that such studies should be done with cardiac muscle, unless special considerations preclude its use. If skeletal muscle were to be used for such studies, however, as in investigations on potassium exchange and content(18,19), the effective concentrations of the drug for the production of the positive inotropic action should be first established on a contracting muscle preparation. The results presented here show that concentrations greater than those required to produce an increased force of contraction are depressant to the diaphragm, and any changes in ionic content of the cells or rate of ionic exchange, or in electrical, metabolic, or other types of activity at these higher toxic concentrations would not contribute to an understanding of the mechanism of the positive inotropic action of the drugs.

Summary. Ouabain increased the force of contraction of isolated guinea pig diaphragm, stimulated directly or indirectly with supra-maximal shocks. At higher concentrations of the drugs, the positive inotropic action was followed by a depressant effect. The concentrations at which these effects occurred were similar to those which produced an increase in force of contraction or toxic effects upon isolated guinea pig ventricular strips. Skeletal muscular paralysis in intact guinea pigs following the administration of ouabain was

frequently not accompanied by evidence of cardiac toxicity. The significance of these findings in relation to studies on the mechanism of action of the cardiac glycosides which employ skeletal muscle preparations was discussed.

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Differential Concentration of Porphyrin in Various Divisions of the Central Nervous System.* (22943)

HARVEY M. SOLOMON[†] AND FRANK H. J. FIGGE

Department of Anatomy, University of Maryland School of Medicine, Baltimore.

The observation that white matter of the central nervous system exhibited a band fluorescence spectrum centered at 625 m led to the conclusion that the nervous system contained coproporphyrin(1). From further evidence based on solubility characteristics, Klüver(2) identified protoporphyrin and coproporphyrin type III isomer. Chu and Watson(3), using the fluorescence quenching technic, also identified the coproporphyrin isomer as type III and reported the average amount of this porphyrin in 10 determinations of normal rabbit brain to be 3.56 γ /100 g brain. The isolation of crystalline coproporphyrin III from beef brain was carried out by Blanshard(4). His method of extraction yielded 2 γ /100 g brain.

Earlier observation,[‡] on the fluorescence of various parts of the central nervous system of mice and rats, gave the impression that red fluorescent material was more abundant in the medulla oblongata and brainstem than in other areas. The quantitative distribution of coproporphyrin III in gross anatomical subdivisions of the central nervous system was

studied to ascertain whether porphyrins exhibit a gradient distribution in the nervous system and to obtain a clue as to the possible functional significance of the nervous system porphyrins.

Methods. Pig brains were collected at the abattoir, stripped of membranes, and washed to remove blood. Equal amounts (250 g) of cerebrum, midgrain, medulla oblongata, cerebellum, and spinal cord were homogenized with 4:1 ethyle acetate-glacial acetic acid, (4) and cooled to -15°C . The supernate was decanted and filtered under suction. Extractions were repeated until no color was observed in the supernate. The material remaining was mixed with Solka-Floc BW 40, a purified wood cellulose filter aid, and placed in a large Büchner funnel, and covered with 200 ml 3N HCl which filtered off slowly. This solution was neutralized with sodium acetate and extracted with ethyl acetate-glacial acetic acid(4:1). The extract was added to the filtered supernate decanted previously. Extractions were carried out 5 times on subdivisions of the central nervous system with exception of the medulla oblongata which was repeated 6 times. The extracted porphyrins were measured fluorimetrically.

Results. Highest concentration of copro-

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[†] Fellow, Nat. F. For Infantile Paralysis.

[‡] Figge, unpublished.

TABLE I. Coproporphyrin III Extracted from Subdivisions of Pig Central Nervous System.

γ of coproporphyrin III/100 g of tissues*					
Cerebrum	Midbrain	Medulla oblongata	Cerebellum	Spinal cord	Total CNS avg
3.05 ± 0.1	$4.23 \pm .27$	$4.85 \pm .31$	3.10 ± 0	$3.16 \pm .22$	3.68

* Mean $\pm$ S.E.

porphyrin occurs in the medulla oblongata (Table I). This is only slightly higher than the amount found in the midbrain. Values for midbrain and medulla oblongata are highest, while values for the cerebrum, cerebellum and spinal cord are slightly lower than the average for the total central nervous system. The average for all 5 subdivisions of the nervous system of the pig is in close agreement (3) with the quantitative determination of coproporphyrin in total rabbit brain (3.56 γ /100 g).

Discussion. Klüver(1) observed the 625 $m\mu$ fluorescence band in funiculi of the spinal cord, and other fiber tracts of the central nervous system. Examination revealed that the 625 $m\mu$ band was absent in peripheral parts of cranial nerves.

Subsequent investigations have been limited to porphyrin content of total brain. Our results indicate that coproporphyrin III concentration varies at different levels of the central nervous system. This observed variation or gradient in coproporphyrin III concentration with a maximum in the medulla oblongata, lends support to the hypothesis that

the central nervous system porphyrins have some functional significance. It should be emphasized that our values represent only the coproporphyrin III content of parts of the central nervous system.

Summary. The central nervous systems of pigs were collected and divided into the various gross anatomical subdivisions. Equal weights of cerebrum, midbrain, medulla oblongata, cerebellum, and spinal cord were extracted for coproporphyrin. A higher concentration of coproporphyrin III was found in the medulla oblongata and brain stem than in cerebrum, cerebellum, or spinal cord. The observed differential coproporphyrin III concentration in various subdivisions of the central nervous system supports the hypothesis that porphyrins in the nervous system may have functional significance.

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Effects of Varying Concentrations of Platelets and Their Lyophilized Derivatives on Generation of Thromboplastin.*† (22944)

E. KLEIN AND R. FIORENTINO. (Introduced by Sidney Farber)

Children's Cancer Research Foundation and Tumor Therapy Group, Children's Medical Center, and Department of Pathology, Harvard Medical School.

It has previously been reported that high concentrations of platelet material, fresh,

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stored at -15°C , or lyophilized, inhibit the coagulation of recalcified, platelet-depleted plasma. Adjustment of the concentration of platelet material to approximate the equivalents of physiological platelet levels, brought the recalcification time into the normal range (1). The nature of these phenomena was

further investigated by examining the effects of varying concentrations of platelet materials in the generation of plasma thromboplastin.

Methods and materials. Fresh and lyophilized platelet concentrates (PC) were prepared as described previously(1,2). Platelet-depleted plasma was prepared by centrifugation at 32,000 g for 30 minutes. Plasma from normal and thrombocytopenic donors was used. A modification of the thromboplastin generation test of Biggs and Douglas(3) was employed. Prothrombin-depleted plasma was prepared by treatment with BaSO₄. The BaSO₄ suspension (40 mg BaSO₄ per cc of plasma) was shaken for 30 minutes and centrifuged at 32,000 g for 30 minutes. The one-stage prothrombin time of this preparation is in excess of 180 seconds. The 0.1 cc aliquots of the incubation mixture were drawn into tuberculin syringes which contained 0.1 cc of a 0.025 M solution of CaCl₂. Contents of syringe were then expelled forcefully into the tube containing substrate plasma. The substrate mixture was rotated manually until the first fibrin strands appeared, which was taken as the end-point. Suspensions of platelet material, varying in concentration between approximate equivalents of 5,000 and 20,000,000 platelets/cu mm, were added to the incubation mixture and the generation of thromboplastin was estimated.

Results. Representative data are summarized in Table I and in Figs. 1 and 2 for fresh and lyophilized platelet materials, respectively.

It can be seen from these data that the

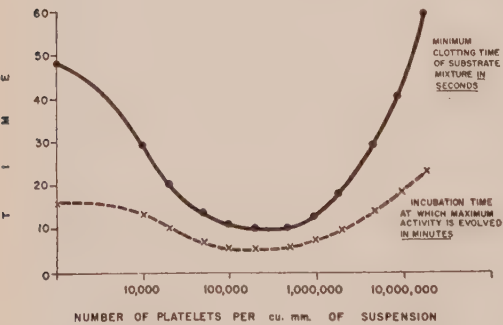


FIG. 1. The Effects of Varying Concentrations of Fresh Platelets on the Generation of Plasma Thromboplastin.

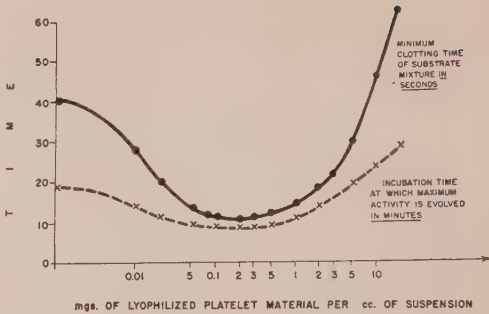


FIG. 2. The Effects of Varying Concentrations of Lyophilized Platelet material on the Generation of Plasma Thromboplastin.

TABLE I. Effects of Varying Concentrations of Platelet Materials on Generation of Plasma Thromboplastin Activity.

No. of platelets or equivalent of suspension	Fresh platelets		Lyophilized platelets	
	Incubation time (min.)*	Min clotting time (sec.)†	Incubation time (min.)*	Min clotting time (sec.)†
× 1000				
20,000	23	59	28	62
10,000	18	40	23	45
5,000	14	29	19	29
2,000	9	18	14	18
1,000	7	13	11	14
500	5	10	9	12
200	5	10	8½	11
100	5	11	8½	11
50	7	14	9	13
20	10	20	11	19
10	13	29	14	28
Control	16	48	19	41

* Time required for incubation mixture to reach maximum thromboplastic activity.

† Clotting time of substrate when incubation mixture has reached maximum thromboplastic activity.

minimum clotting time and the incubation time, at which the corresponding maximum activity develops, vary in approximately the same manner with analogous concentrations of the 2 types of platelet preparations. At high concentrations the generation of plasma thromboplastin activity is inhibited. Normal values are attained at the approximate equivalent of physiological concentrations.

Discussion. It is apparent from the data presented here that generation of plasma thromboplastin activity is related to amount of platelet material in the incubation mixture. The amount of platelet material determines the maximum thromboplastin activity, as

measured by the minimum clotting time of the substrate, and the rate at which that level of activity is developed.

The inhibitory effects of high concentrations of platelet materials on recalcification time(1) are paralleled by inhibition of the generation of plasma thromboplastin. The mechanism of the inhibitory phenomena is obscure, but there are indications that platelets may contain specific activities which inhibit coagulation(4,5,6). Platelet materials do not activate the generation of thromboplastin excessively at any concentration. Extracts(4) and fractions of platelet material behave similarly in regard to inhibition and to the absence of excessive activation of coagulation of varying concentrations of the respective platelet preparations.

These *in vitro* studies, therefore, do not present contraindications to the *in vivo* administration of such materials in regard to the hypothetical hazards of thromboembolic phenomena. In parallel animal and clinical studies here(7,8) and in animal studies by others(9) no evidence for hyper- or hypo-coagulation has been obtained after intravenous infusions of large amounts of various types of platelet preparations.

It can be seen that lyophilized platelet material produces approximately the same degree of activation and inhibition as fresh platelets at corresponding concentrations. The lyophilized material, however, requires longer periods of incubation than fresh platelets for reaching maximum levels of thromboplastin.

The effects described here may play a role in the hemorrhagic phenomena encountered in some cases of idiopathic thrombocythemia† and in the thrombocythemia secondary to polycythemia, leukemia, the post-splenectomy state and others.

Summary. High concentrations of fresh and lyophilized platelet materials inhibit the generation of plasma thromboplastin. Lyophilized platelet material, at the approximate equivalent of physiological concentrations, produces a normal level of plasma thromboplastic activity. Thromboplastin generation is not activated excessively at any concentration of these preparations.

† Personal communication.

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Serum Aldolase Activity in Neuromuscular Disorders. 2. Experimental Application.* (22945)

STANLEY M. ARONSON AND BRUNO W. VOLK

Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn, N. Y.

Extensive clinical application of the serum aldolase determination has demonstrated its value in the serologic diagnosis of progressive muscular dystrophy(1,2,3). Muscle atrophies secondary to inanition, disuse or paralysis of cerebral origin have not caused any serum elevation of this enzyme. Muscle atrophy consequent to a lower motor neuron deficit (e.g., recently convalescent poliomyelitis or infantile progressive spinal muscular atrophy) appears to result in a moderate, occasionally evanescent, rise in the serum aldolase, never however to the degree representative of progressive muscular dystrophy. These clinical observations suggest that serum aldolase may reflect atrophic changes due to neuromuscular junction deficit as well as those caused by primary myopathies.

In an effort to substantiate experimentally these findings a series of 33 dogs were submitted to various procedures resulting in muscle atrophy.

Materials and methods. A. 13 mongrel dogs, weighing from 15 to 20 kg each, were subjected to a femoral nerve section, performed in the upper inguinal canal. B. Ten dogs were subjected to an achilles tendon section or suprapatellar tenotomy. C. Four dogs were submitted to a lumbar laminectomy following which the cauda equina roots and the sacral cord were surgically transected. D. In 3 dogs, the left femoral artery was surgically constricted in the upper inguinal canal. E. Three dogs were submitted to a sham surgical procedure in which the inguinal canal was entered but in which neither the traversing femoral nerve nor artery were violated. Pre-operative serum was withdrawn on all dogs to establish the normal levels of serum aldolase, determined by the technic of Sibley and Lehninger(4) and SGO-trans-

aminase, using the procedure described by Karmen, Wroblewski and La Due(5). At intervals of 3 to 7 days following surgical intervention, serum for these evaluations was withdrawn from all dogs for periods up to 210 days. In addition, periodic biopsies of the involved musculature as well as normal muscle tissue derived from an uninvolved limb were carried out. Resected muscle biopsies were studied histologically to determine the degree of observable atrophy, quantitated by means of measurement of the average muscle fiber diameter microscopically, and the remaining fresh tissues used to determine the level of tissue aldolase and phosphorylase. Tissue aldolase levels were derived by the method described by Sibley and Lehninger (4). The technic described by Cori and associates(6) for the determination of tissue phosphorylase b was used. The values of phosphorylase b were utilized in accordance with the greater reliability of this fraction(6). At the termination of all experiments the dogs were autopsied for verification of the procedures employed.

Results. A. *Histologic studies:* An unremitant atrophy of the involved skeletal musculature was noted following neurotomy, tenotomy, cauda equina transection or vascular occlusion. Muscle fibers displayed a significant diminution in cross sectional diameter, assumed a slightly basophilic appearance and showed a relative increase in sarcolemmal cells. Cross striations, however, were invariably preserved and no fatty infiltration, significant fibrosis or necrosis was observed. The most extensive atrophic alterations were associated with animals subjected to cauda equina transection (Group C) (Fig. 1).

B. *Serum aldolase and SGO-transaminase:* In all categories, including the animals comprising the sham surgery group, a variable degree of elevation in serum aldolase was re-

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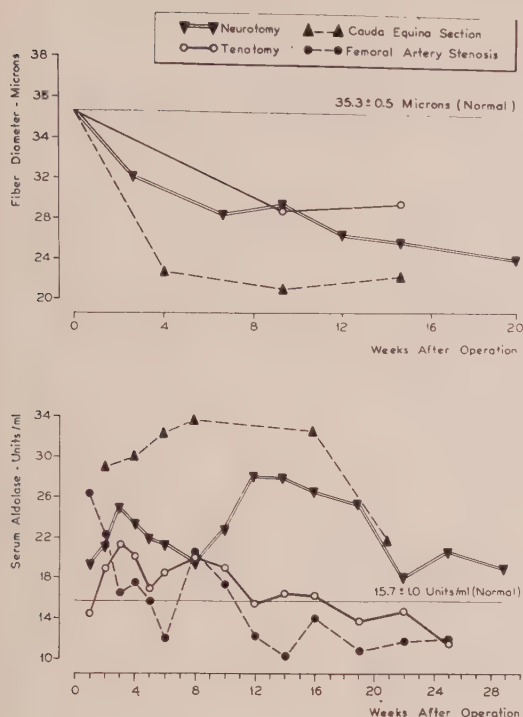


FIG. 1. Effect of various forms of experimentally induced muscle atrophies upon muscle fiber diameter (above) and serum aldolase (below).

corded in the 2 weeks following surgery. A plateau within normal limits ($15.7 \text{ units/ml} \pm 1.0 \text{ S.E.}$) was then achieved in the tenotomy, femoral artery constriction and sham-operated dogs (Groups B, D and E). In the animals which have undergone neurotomy (Group A) a second elevation of serum aldolase was seen commencing about 9 weeks after nerve section reaching levels of $28.0 \text{ units/ml} (\pm 2.7)$, finally reverting to normal approximately 21 weeks after the experimental procedure. This delayed elevation was noted in 10 of the 13 dogs constituting this group. Weekly serum aldolase levels of the dogs in which multiple motor roots were divided within the vertebral canal (Group C) showed a sustained rise beginning immediately after the operation and reaching the highest concentrations of this series, reaching levels of $33.7 \text{ units/ml} (\pm 3.3)$ (Fig. 1). Some regression of the hyperaldolasemia was seen in this group by the 18th week after surgery. No

elevations of SGO-transaminase were observed except for an insignificant and transient post-operative rise in some dogs.

C. Tissue phosphorylase and aldolase: A wide fluctuation in tissue enzyme levels was noted. Nevertheless a progressive but slight decrease in the tissue phosphorylase b from normal mean values of $20,267 \text{ units/g}$ of non-collagenous protein ($\pm 1,725$) was seen in the 7 to 8 weeks following neurotomy (Group A). In the succeeding 7 weeks a sharp fall in tissue phosphorylase levels to a mean of $8,584 \text{ units/g}$ of non-collagenous protein was demonstrable. The range, however, was still wide, varying from $2,620$ – $28,500 \text{ units/g}$. A roughly similar pattern of decreased enzyme activity was noted in the determined levels of tissue aldolase in animals subjected to neurotomy. The normal mean value was 23.1 units/g of non-collagenous protein $\times 10^4$ (± 1.1). Twenty weeks after operation a mean of 15.3 units (range: 13.6 – 18.2) was obtained. In contrast, no depression of either the phosphorylase b or the aldolase in involved tissues was seen in animals in which tenotomy (Group B) was performed. Animals submitted to the more extensive neuromuscular atrophy secondary to cauda equina section (Group C) showed a depression of both tissue enzymes. Five weeks after operation the mean tissue aldolase was 19.2 units/g of non-collagenous protein $\times 10^4$ (range: 18.7 – 19.8 units) and the phosphorylase b, $14,048 \text{ units/g}$ of non-collagenous protein (range: $2,145$ – $31,600 \text{ units}$).

Discussion. The progress of various forms of experimental muscle atrophy was surveyed in terms of serial histologic and enzymatic assay. No significant distinction between the atrophy induced by neurotomy, cauda equina section, tenotomy or vascular constriction could be established on the basis of microscopic appearance alone. In all instances, a progressive decrease in muscle cell diameter was noted, with preservation of cross striations. No singular morphologic feature emerged to distinguish one form of atrophy from another.

In dogs submitted to femoral neurotomy

(Group A) or the more extensive denervation resulting from transection of the cauda equina nerve roots (Group C), the levels of skeletal muscle aldolase and phosphorylase b diminished in accordance with previous observations of others(7). Two rises in serum aldolase were seen in this series of dogs. The first immediately following the surgical procedures was attributed to the operative intervention and resulting tissue damage, and was noted also in dogs subjected to sham-operation without compromise of the motor nerve supply. The initial post-operative elevation was slightly higher in the neurotomy group as compared to the animals subjected to tenotomy, possibly because of the more extensive tissue trauma incident to the former procedure. The second, delayed elevation occurred about 9 weeks after neurotomy, persisted for about 13 weeks and then reverted to normal. Previous investigators have surmised that elevated serum aldolase, in the absence of hepatic disease or extensive tissue necrosis, may be due to liberation of this enzyme from degenerative muscle(8,9). A delayed rise of this serum enzyme, exhibited by dogs in which motor nerves have been severed, would therefore be anticipated, if an inverse correspondence between serum and tissue aldolase is accepted. Further, the quantitative extent of neuromuscular atrophy seems to bear some relationship to the serum aldolase rise. Animals in which the cauda equina roots were sectioned showed the greatest topographic degree of muscular atrophy and the most pronounced and prolonged incrementation of this serum enzyme. It is to be emphasized, however, that the transient serum aldolase elevation seen in these nerve-section dogs is not as sustained nor as marked as the hyperaldolasemia noted in human cases of muscular dystrophy(3).

A simulation of disuse atrophy was induced in dogs by severing the tendons of major lower limb muscle groups. Atrophy, as exemplified by morphologic study of serial muscle biopsies, advanced progressively, but no alteration in serum aldolase was noted during the prolonged period of observation. Tissue phosphorylase b remained essentially un-

altered in the face of this continuing atrophy and tissue aldolase was not diminished, in contrast to the changes of these enzymes seen in animals in which nerve section was performed. In some instances, the tissue aldolase levels appeared to be actually elevated above normal. This finding was interpreted as indicating only a relative increase of enzyme content attributable to the decrease of tissue bulk during the course of inactivity.

Atrophy consequent to vascular anoxia did not appear to affect the level of the serum aldolase, during the period of observation, except during the initial post-operative period.

SGO-transaminase determinations in all groups failed to show any significant elevation other than some occasional transient rises immediately after surgery.

Summary. Thirty-three mongrel dogs were submitted to a variety of experimental procedures instituted for the purpose of creating various forms of experimental muscle atrophies. Dogs in which either the femoral nerve was sectioned or the entire cauda equina root system was severed showed a delayed and erratic depression of tissue phosphorylase b and aldolase, and in parallel fashion, a delayed but transient rise in the serum aldolase. Dogs in which the atrophy was caused by tendon section or compromise of the arterial blood supply, showed no depression of the tissue enzymes or commensurate elevation of the aldolase level within serum. The degree of atrophy, as determined by histologic measurement of the implicated muscle fibers, however, was approximately equivalent in all groups. These findings are in concurrence with the clinical data accumulated in the application of the serum aldolase test and appear to indicate that atrophy consequent to denervation may be distinguished from atrophy of other causes by the moderate and transient rise in the serum aldolase.

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In vivo Effect of CoA and Other Factors on Lipide Phosphorylation.* (22946)

W. E. CORNATZER AND FRED SNYDER

*Guy and Bertha Ireland Laboratory, Department of Biochemistry, University of N. Dakota
Medical School, Grand Forks.*

Considerable progress has been made in possible pathways of phospholipide synthesis in liver for partial purified enzyme systems. Recent work has provided evidence that CoA (1), ATP(2,3), and maintenance of oxidative phosphorylation(4) are essential for phospholipide synthesis in liver mitochondria. Recently Kennedy has shown that liver mitochondria synthesize lecithins, involving phosphorylcholine as an intermediate and incorporate choline directly into the lipid molecule, requiring ATP and CTP(4,5). Phospholipide synthesis in the liver of animals maintained on stock diet as measured from P^{32} turnover studies is already at a maximum rate(6) and even under nutritional stress or liver cell death this is not altered. In choline deficiency the total liver lecithin content decreases and total lipid concentration increases(7) resulting in increased need in the liver cell for choline-containing phospholipides(8). Thus, compounds which are involved directly in phospholipide synthesis should affect the rate of turnover in fatty liver cell since there is an increased demand for lecithins. In view of these observations and the experimental work on partial purified enzyme systems, the *in vivo* effects of CoA, ATP, AMP, oxalacetate and CMP on lipid phosphorylation in liver of rats have been investigated in choline deficient animals.

Methods. Male albino rats of Sprague-

Dawley strain weighing 100-110 g were divided into 5 series. The animals in Series I, II, and III were maintained on 5% Casein-5% Fat Diet(9), for 4 weeks. In Series IV, rats were maintained on pantothenic acid deficient diet(10) for 10 weeks. Control animals for this group received similar diet containing the vitamin. In each series, 4 to 6 rats were housed in individual cages with raised screen bottoms. Animals were fed *ad libitum*. Intraperitoneal injection of 4 μ c of P^{32} as NaH_2PO_4 was administered one minute following injection of above compounds listed in Series I-IV. Six hours later the animals were killed by decapitation. The liver was removed, rapidly weighed and divided into 2 fractions. On one fraction acid-soluble phosphorus was removed by extracting with 10% TCA containing 0.4 M $MgCl_2$ (11) and radioactivity and total P(12) were determined. The other fraction was covered with alcohol and lipides extracted with alcohol, alcohol-ether and purified with chloroform (13). On aliquots of the chloroform solution, radioactivity(13) and phosphorus(12) were determined. As a measure of phospholipide synthesis specific and relative specific activity (14) have been calculated. Twelve animals in Series V were maintained on Diet 26(9) for 8 weeks. At the time of dietary regime they were divided into 3 groups and intraperitoneal injection of compounds listed in Series II(5) was given. One minute later 4 μ c of P^{32} was administered and in 6 hours the animals were killed by decapitation, liver was

*Supported by grants from Lipotropic Research Foundation and Atomic Energy Commission.

TABLE I. Effect of a Single Dose of Various Agents on Lipide Phosphorylation in Liver of Rats.

Series No.	Group No.	Materials admin.	No. of animals	Relative specific activity of phospholipide P
I	1	CoA	10	.154 ± .023
			17	.221 ± .030†
	2	AMP	10	.162 ± .033
			15	.172 ± .022
	3	AMP	5	.155 ± .037
		ATP	5	.186 ± .018
		ATP*	5	.162 ± .030
	4	Ox†	4	.162 ± .028
			10	.192 ± .024
			8	.180 ± .024
II	1	Choline " , CoA	6	.160 ± .025
			7	.230 ± .027†
	2	Choline " , Ox	7	.217 ± .042†
			5	.182 ± .020
	3	Choline " , AMP	6	.171 ± .023
			4	.218 ± .050
			5	.200 ± .013
	4	Choline " , AMP, Ox	5	.278 ± .026†
			5	.257 ± .037
			9	.158 ± .030
	5	Choline " , Ox, AMP, CoA	9	.164 ± .015
			9	.224 ± .025†§
			18	.161 ± .018
III	1	Choline	19	.202 ± .046†
		" , Ox	20	.289 ± .018†§
		" , " , choline		
		" , " , choline		
		" , " , choline		
	2	CMP	5	.124 ± .013
		CMP, CoA	4	.143 ± .019
		" , " , AMP, Ox, choline	4	.167 ± .023†
		" , " , AMP, Ox, choline	5	.189 ± .019†§
		" , " , AMP, Ox, choline	5	.161 ± .024
IV	1	Ca pantothenate†	5	.179 ± .012†§
			6	.166 ± .032
			5	.148 ± .022
			4	.246 ± .052
			3	.219 ± .011
	2	Ca pantothenate†	5	.124 ± .013
			4	.143 ± .019
			4	.167 ± .023†
			5	.189 ± .019†§
			5	.161 ± .024

All materials inj. as 10 mg/100 g body wt, except CoA and CMP 5 mg and oxalacetate 50 mg. Controls received equivalent amounts of saline.

No. preceded by ± are stand. dev.

* 100 mg/100 g body wt.

† Ox = Oxalacetate.

Test of significance was applied to difference between means of controls, saline (‡) or choline (§), and experimental values. The P probability for chance occurrence of this difference was <.01; || indicates that this probability when compared to saline was <.05.

removed and minced in an iced beaker, diluted 10 fold with cold 0.88 M Sucrose. The mince was homogenized in Potter-Elvehjem

type tissue grinder with teflon pestle for 4 minutes in ice bath and centrifuged in Servall Angle Centrifuge (Type PR-1), according to modification of procedure of Hogeboom *et al.* (15) and Griffiths and Pace (16) for separation of cellular fractions by differential centrifugation. Total lipides from cellular fractions were extracted with 95% ethanol for 6 hours in Soxhlet Continuous Extractor. The lipides were purified with chloroform (13). After evaporation of solvent, the residue was dissolved in methanol and the solution filtered and treated with MgO for separation of choline-containing from non-choline-containing phospholipides (17). Radioactivity and phosphorus were determined on aliquots of the solution before and after absorption on the MgO.

Results. The effects of CoA and other factors on lipid phosphorylation are shown in Tables I-III. To evaluate the statistical significance of the results, the *t* test of significance (18) was applied to the difference between means for controls receiving saline or choline and experimental values. It is apparent that administration of a single dose of CoA stimulates lipid phosphorylation as shown from data of Series I. This stimulating effect is still pronounced in those animals of Series IV which are pantothenic acid deficient where CoA synthesis is limited. This increase of lipid phosphorylation due to ad-

TABLE II. Effect of CoA and Pantothenic Acid on Lipide Phosphorylation in Pantothenic Acid Deficient Rats.

Series No.	Materials admin.	Exp. conditions	No. of animals	Relative specific activity
IV	NaCl	Controls	15	.127 ± .02
		Deficient	7	.153 ± .01
	CoA*	"	9	.180 ± .02†§
		Controls	17	.144 ± .02
	Ca pantothenate†	Deficient	10	.166 ± .025
		"	9	.156 ± .022

* 10 mg/100 g body wt.

† 2.65 mg/100 g body wt, or .013 mole of pantothenic acid.

The *t* test of significance was applied to difference between means of saline controls (‡), or saline deficient (§) and experimental values. P probability for chance occurrence of this difference was <.01.

No. preceded by ± are stand. dev.

TABLE III. Phospholipide Synthesis in Cellular Fractions of the Liver, 4 Samples/Group.

Series No.	Materials admin.	Specific activity of phospholipide P					
		Mitochondria		Nuclei		Homogenates	
		c.c.	n.c.c.	c.c.	n.c.c.	c.c.	n.c.c.
V	Saline	80.62	60.69	81.90	60.62	83.65	78.02
	Choline	85.95	37.96	90.80	41.26	92.31	94.30
	Choline, oxalacetate, AMP and CoA	111.34	64.80	101.73	57.58	107.54	72.53

c.c. = choline containing phospholipides. n.c.c. = non-choline containing phospholipides.
All materials inj. as 10 mg/100 g body wt, except CoA 5 mg and oxalacetate 50 mg.

ministration of CoA is not the result of pantothenic acid content of CoA, for animals of Series V which received single injection of vitamin did not demonstrate any stimulation. This would be in agreement with the data of Chernick *et al.* (19) who have shown that CoA content of rat liver is not limited by availability of pantothenate and that injection of calcium pantothenate had no effect on liver CoA content.

Administration of AMP, or ATP had no effect on stimulation of lipid phosphorylation. This may be due to rapid hydrolysis of these compounds following injection. Oxalacetate administration stimulates lipid phosphorylation as shown from data of Series I. The dose of choline used to stimulate lipid phosphorylation which the animals received in Series II is considerably less than that previously reported (6). This dose was chosen so that the effect of other agents when used in conjunction with choline would be demonstrable. It is apparent from data of Series II that there is a more marked lipid phosphorylation when CoA, AMP and oxalacetate is mixed with choline than when choline or saline alone was administered. This mixture stimulates lipid phosphorylation in both choline and non-choline-containing phospholipides in the mitochondria and nuclei of the liver as shown from data in Table III. The greatest stimulation occurred in the lecithin fraction. It is apparent from data in Series III that CMP stimulates lipid phosphorylation. There is further stimulation of lipid phosphorylation when oxalacetate, AMP and choline is mixed with CMP. These results demonstrate clearly that CoA, oxalacetate and CMP stimulate lipid phosphorylation and give further *in vivo* support to their role in

purified enzyme system (1,4,5).

Summary. 1. The *in vivo* effects of CoA, ATP, AMP, CMP and oxalacetate on lipid phosphorylation in the liver of rats have been investigated in choline deficient rats. 2. A single dose of coenzyme A stimulates phospholipide synthesis in choline and in pantothenic deficient animals; oxalacetate stimulates in choline deficient animals. 3. Administration of a single dose of a mixture of CoA, AMP, oxalacetate and choline stimulates lipid phosphorylation in both mitochondria and nuclei greater than choline or saline alone. The greatest stimulation occurred in the lecithin fraction. 4. Cytidine monophosphate stimulates lipid phosphorylation. There is further stimulation when CMP is administered with AMP, oxalacetate and choline.

The following abbreviation will be used in this paper: CoA = Coenzyme A; ATP = adenine triphosphate; CTP = cytidine-5'-triphosphate; AMP = adenosine-5'-phosphate; CMP = cytidine-5'-monophosphate; TCA = trichloroacetic acid.

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Ammonia Excretion and Renal Glutaminase Activity During Administration of Strong Acid and Buffer Acid.* (22947)

DONALD W. SELDIN, H. C. TENG, AND FLOYD C. RECTOR, JR.
(Introduced by Carleton B. Chapman)

Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas.

Excretion of urinary ammonia rises during administration of strong acid loads as a consequence of increased ammonia production owing to activation of the ammonia-producing enzymes of the renal tubular cell(1,2,3) and accelerated ammonia transport because of enhanced diffusion of ammonia into an acid urine. Activation of renal glutaminase and other ammonia-producing enzymes by administration of a strong acid (NH_4Cl) could result from the impact of the acid load on intracellular buffers, effect of acid tubular fluid, or rapid removal of ammonia from the tubular cell.

Theoretically, a buffer acid load should have the same effect on the intracellular pH as a physiologically equivalent load of strong acid; in the kidneys, however, the presence of the buffer anion permits secretion of large amounts of H^+ without a drastic fall in urine pH. Ingestion of NaH_2PO_4 , therefore, accelerates excretion of titratable acid without notably altering ammonia excretion(4,5). In the present study, therefore, the effects of physiologically equivalent loads of strong acids and buffer acids on ammonia excretion and renal glutaminase activity were compared

to determine whether activation of glutaminase results from prerenal or renal effects of an acid load.

Procedure. Three groups of male Sprague-Dawley rats, weighing 200-300 g, were fed 10 cc of standard electrolyte deficient diet (SEDD) twice daily and distilled water *ad lib.* for 14 days (Table I). Group I received only the SEDD, Group II the SEDD plus 2 mM KCl and 3 mM NH_4Cl daily, Group III the SEDD plus 2 mM KCl and 3.75 mM NaH_2PO_4 .† Daily 24 hour urine samples were collected under mineral oil using phenyl mercuric nitrate and toluene as preservatives. The ammonia of urine was determined by modification of the microdiffusion method of Conway(6). Urine pH was measured with a Beckman pH meter using external glass electrodes, and corrected to 37.5°C by a factor of 0.01 pH unit per degree. To determine titratable acid, urine was titrated to a corrected pH 7.4 with 0.1 N NaOH using Beckman automatic titrator. At the end of

† Since the pK of NaH_2PO_4 is 6.8, the ratio $\frac{\text{Na}_2\text{HPO}_4}{\text{NaH}_2\text{PO}_4}$ at pH 7.4 is $\frac{4}{1}$. Therefore, 3.75 mM of NaH_2PO_4 would have the same impact on the buffer systems of the body as 3 mM NH_4Cl , since only 3 mM of the NaH_2PO_4 would be converted to Na_2HPO_4 .

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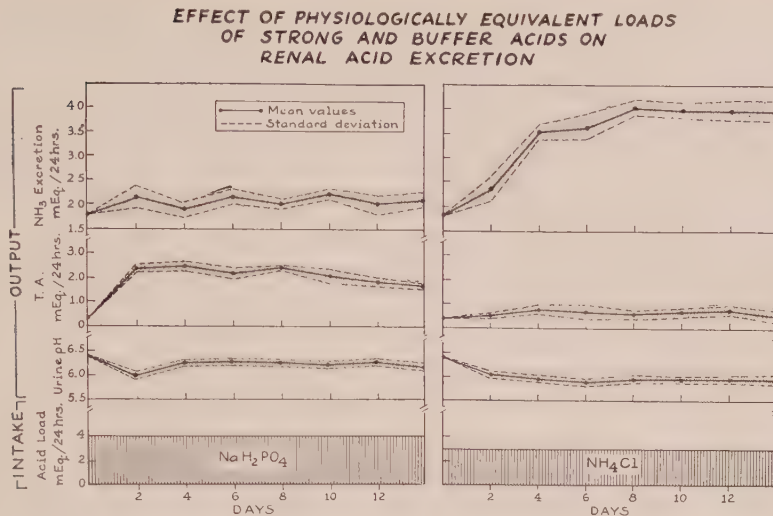


FIG. 1.

the experimental period the rats were anesthetized by intraperitoneal injection of sodium amytal and arterial blood collected anaerobically from the abdominal aorta. Kidneys were removed and immediately frozen. Maximal glutaminase activity was measured in a cold homogenate of kidney using the method previously described(2). The CO_2 content of blood was measured on 0.2 ml serum(7).

Results. Daily urinary acid excretion in control rats maintained on SEDD alone remained constant throughout a 14 day period, amounting to 1.7 ± 0.2 mEq for ammonia, 0.4 ± 0.1 mEq for titratable acid and 6.4 ± 0.2 for pH.

During administration of NH_4Cl loads, excretion of urinary ammonia rose slowly, reaching a peak in 4 to 6 days (Fig. 1). Urine pH fell during the first two days to approximately 5.9, but titratable acid did not change significantly. The rise in urinary ammonia was accompanied by an increase in renal glutaminase activity (Table I).

Physiologically equivalent loads of NaH_2PO_4 , on the other hand, produced no change in ammonia excretion, while titratable acid rose sharply despite a very slight fall in urine pH (Fig. 1). Renal glutaminase activity remained unchanged (Table I).

Despite the marked difference in their effects on the pattern of acid excretion and the

activity of renal glutaminase, NH_4Cl and NaH_2PO_4 loads produced approximately the same slight depression of serum bicarbonate (Table I).

TABLE I. Effect of NaH_2PO_4 Loads on Serum Bicarbonate Concentration and Renal Glutaminase Activity.

Treatment	Serum HCO_3^-	Renal glutaminase, μM $\text{NH}_3/100$ mg dry kidney/hr
Standard electrolyte deficient diet (SEDD)	23.9 ± 1.2	788 ± 59
SEDD + 2 mM KCl + 3 mM NH_4Cl daily	19.6 ± 1.4	1850 ± 100
SEDD + 2 mM KCl + 3.75 mM NaH_2PO_4	20.5 ± 1.6	675 ± 69

Conclusions. Activation of renal glutaminase during administration of NH_4Cl does not appear to result from pre-renal effects of the acid load, since physiologically equivalent loads of NaH_2PO_4 produced the same slight depression of serum bicarbonate but affected neither excretion of ammonia nor activity of renal glutaminase. Rather, it appears that activation of glutaminase is related to changes, as yet unidentified, within the kidney.

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A New Simple Method for Indirect Determination of Blood Pressure in the Rat.* (22948)

CARL S. ALEXANDER.† (Introduced by E. B. Flink)

Department of Medicine, University of Minnesota Medical School, Minneapolis.

Technics generally used for indirect determination of blood pressure in the rat's tail have one or more of the following disadvantages: 1) they are cumbersome, 2) they require complex apparatus, or 3) they are relatively inaccurate. The older plethysmographic methods(1,2,3) have been further complicated by adapting them to electronic devices in order to increase their sensitivity (4). The photoelectric tensometer depends upon change in transmission of a light beam incident on a small area of the rat's tail(5) or foot(6). Friedman and Freed(7) described what is called a microphonic manometer which, according to these observers, operates by amplifying the pulsatile vibrations of the caudal artery in the tail similar to that noted in the indirect recording of blood pressure in the human brachial artery. A graphic method of recording these tail vibrations has been reported to yield greater accuracy than that obtained with the microphonic manometer(8).

The present report is concerned with the relationship of these pulsatile vibrations, as seen by the naked eye, to blood pressure in the rat's tail as measured by other standard procedures. Thus, by simply observing the characteristic pattern of vibration in the tail, the indirect systolic pressure may quickly be determined without the use of expensive or

complex equipment. This report also deals with comparative measurements of blood pressure after whole body warming versus tail warming, the influence of cuff length on blood pressure readings, and difficulties encountered following the use of vaso-constrictor agents.

Materials and method. 1. Description of the Visual Method. When the rat's tail is warmed for 5-10 minutes or the rat itself is placed in a ventilated incubator at 39°C for 15 minutes and a 20 mm cuff attached to a manometer is placed on proximal part of tail, pulsatile vibrations can readily be seen along the entire length of the tail. These movements are of maximal amplitude at the distal end of the tail which must be allowed to hang freely. If the cuff is then inflated to a pressure exceeding the systolic pressure, the vibrations cease. With slow deflation of the cuff the vibrations suddenly reappear and increase in amplitude as the cuff pressure approaches zero. The pressure at which the pulsations make their first reappearance is taken as the systolic endpoint. This point is sharp, reproducible within narrow limits, and easily seen by the inexperienced observer. Often the endpoint is heralded by an arching upward of the end of the tail, after which the vibrations immediately appear. Observations of minimal vibrations are facilitated by use of darkened room with a lamp placed so that a sharp shadow of the tail is cast on a white background one or two cm behind the tail. To avoid struggling and to limit extraneous movements, the animals are confined in a

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† Formerly Cardiac Trainee of U.S.P.H.S. Present address: Veterans Admin. Hospital, Minneapolis, Minn.

TABLE I. Blood Pressure Determination in Normal and Hypertensive Rats by 4 Different Methods.

Wt range, g	No. of rats	Visual method*		Audiometric method*		“Cut tail” method†		Aortic mean‡	
		Avg	Range	Avg	Range	Avg	Range	Avg	Range
mm Hg									
Normotensive rats									
200-275	24	115 ± 10.6	92-132	112 ± 10.4	92-131				
275-350	20	125 ± 10.9	108-140	122 ± 16.4	103-158	117 ± 13.7	90-135	88 ± 19.7	62-113
350-475	20	124 ± 9.7	108-142	119 ± 11.5	100-143	121 ± 10.8	100-140	104 ± 14.6	75-122
Hypertensive rats§									
335-444	9	165 ± 16.9	143-187	151 ± 18.8	130-183	153 ± 17.7	130-180	149 ± 9.3	136-166

* 4-6 observations/rat by visual and audiometric methods.

† Single observation/rat.

‡ " " " at time of sacrifice under light ether anesthesia.

§ Animals subjected to prolonged desoxycorticosterone trimethylacetate administration and increased salt intake.

container during the study. Suitable containers may be made simply and inexpensively by telescoping 2 empty tin cans from which the bottoms have been removed but with the conical tops left intact. Different sized cans may be selected to accommodate virtually any size rat. Measurements should be made immediately after removal from incubator since cooling results first in diminution and later complete disappearance of the vibrations. Pulsations reappear upon re-heating the animal or its tail.

2. Experimental procedures. The blood pressure was determined in male rats of the Sprague-Dawley strain fed a fox chow diet *ad lib*. Their weights ranged from 200-475 g. Measurements were made as described previously. Where measurements were made with the microphonic manometer, the microphone was placed just distal to the cuff allowing the rest of the tail to hang freely. Simultaneous measurements were made by 2 observers independently, one recording the blood pressure by the visual method as noted above, while the other employed the microphonic manometer (audiometric method) essentially as described by Friedman and Freed(7).‡ In a preliminary experiment involving 13 normal rats, blood pressure measurements by the visual method were made with and without the microphone suspended from the tail to determine if weight of micro-

phone dampened the vibrations. In no instance did the readings differ significantly. In 3 of the 4 groups of normal rats, 2 additional methods were used to measure blood pressure as soon as visual and audiometric readings were completed. With the cuff inflated beyond the systolic pressure level as previously determined, the distal 2-3 mm of the tail was amputated with a sharp scissors. The cuff was then slowly deflated until the first drops of blood spurted from the cut end. This was recorded as systolic pressure by the "cut tail" method. Blood loss was minimized by securing prompt hemostasis with an elastic band positioned just proximal to the cut end. The animal was then removed from the container, lightly anesthetized with ether, and the abdomen was opened along a mid-line incision. A 20 gauge needle was inserted into the abdominal aorta at its bifurcation and the pressure was recorded with the aid of a Statham strain gauge transducer connected to a Sanborn recorder. The latter was equipped with an electrical integrator which was used to obtain direct mean aortic pressure. Limitations of the recording apparatus available did not permit accurate measurement of systolic pressures. Two cuffs, 20 and 7 mm in length, were designed for comparison of blood pressure values as a function of compressed tail length. The cuffs were made of one half inch Penrose drain incorporated into a rigid receptacle allowing for inflation with air. The cuff was connected to either a mercury or aneroid type

‡ An additional amplifier permits more accurate readings. In some cases a Stethotron was used in conjunction with apparatus.

TABLE II. Influence of Cuff Size on Blood Pressure Measurement.

Wt, g	Cuff size, mm	Visual method*		Audiometric method*		"Cut tail" method†
		Avg	Range	Avg	Range	
330	20	145	144-145	140	140-140	135
	7	230	220-240	210	200-220	
310	20	134	132-136	154	145-158	118
	7	175	175-175	192	185-195	
326	20	130	126-135	136	134-138	135
	7	150	145-152	151	150-152	
286	20	133	130-136	134	132-135	140
	7	173	170-175	167	164-170	
323	20	138	134-144	139	136-144	140
	7	175	170-180	174	170-178	
	20	147	145-148	155	152-158	
340	20	114	108-120	142	140-144	125
	7	183	180-185	182	180-184	
	20	123	115-130	146	144-148	
305	20	128	125-130	130	126-132	116
	7	188	185-190	182	178-185	
	20	125	125-130	127	122-132	
288	20	114	110-118	110	108-112	115
	7	150	135-160	—	over 300	
	20	123	120-125	121	117-125	
272	20	119	118-120	101	95-108	118
	7	134	125-140	142	140-144	
	20	125	124-125	108	105-111	

* Avg of 2-3 observations/rat.

† Single observation.

manometer. The experiments in which only the tail of the animal was heated were performed a few days after the blood pressures were determined in the usual way, *i.e.* after the animal had been kept in an incubator for 15-20 minutes at 39°C. The tail was heated by means of a light bulb situated at a variable distance from it and measurements were made as soon as vibrations appeared.

Results. 1. Comparison of indirect and direct methods. As shown in Table I, blood pressures determined by the 3 indirect methods—visual, audiometric, and "cut tail method" were approximately the same for

normal rats weighing 200-475 g. The direct aortic mean pressure was definitely lower than the value obtained by indirect methods in the case of normal rats. In the hypertensive range, however, the direct measurements approximated those obtained by indirect means. Two other observations are of special interest: 1) In comparative studies in which movement of the tail was almost completely prevented by holding the tip of the tail gently with a forceps the microphonically audible sounds disappeared or were markedly diminished. 2) In some rats systolic pressure could not be determined with the microphonic technic because the sound persisted with no change in its character up to pressures of 300 mm of mercury.

2. Blood pressure measurements as a function of cuff size. As seen in Table II, visual and audiometric blood pressure readings obtained using the 7 mm cuff far exceeded those obtained with the 20 mm cuff in every case. However, in the case of the 3 rats studied with the "cut tail method" blood pressure values were independent of cuff size.

3. Whole body warming versus tail warming. It is evident from Table III that blood pressure readings were almost the same regardless of which warming method was used.

4. Studies with epinephrine. Each of 5 rats was injected subcutaneously with 0.2 mg of epinephrine in oil. Blood pressure readings by the indirect methods were obtained 30 minutes and 2 hours after the injection after preliminary whole body warming at 39°C for 15 minutes. Seven other animals received 0.05 mg of aqueous epinephrine subcutaneously and were then warmed in a similar manner. Blood pressure readings were made at 15-minute intervals for 2 hours. No sig-

TABLE III. Blood Pressure Measurements after Whole Body and Tail Warming.*

Wt, g	Visual method				Audiometric method			
	Body warming		Tail warming		Body warming		Tail warming	
	Avg	Range	Avg	Range	Avg	Range	Avg	Range
204	116	110-122	112	110-115	107	100-118	107	104-110
212	120	114-125	118	112-120	118	112-124	114	110-116
214	122	112-130	121	116-126	115	105-122	115	114-118
233	104	104-104	114	110-118	98	95-100	116	110-118
249	113	110-120	123	120-125	107	106-108	120	118-122

* Avg of 2-3 observations/rat.

nificant effect on blood pressure was noted in either of these experiments. The experiment was then repeated using aqueous epinephrine in doses of 0.2 mg subcutaneously. This resulted in extreme excitability and marked blanching of the peripheral parts including the ears, feet, and tail. Despite warming, tail vibrations were not visible, and no sound could be heard with the microphonic manometer.

Discussion. Measurement of rat blood pressure by the visual observation of tail vibrations as a systolic endpoint as described herein has the advantage of simplicity, low cost, and accuracy when compared to other indirect methods.

Since the sounds heard with the microphonic manometer are abolished or markedly decreased by holding the tail rigidly in position, it is apparent that these sounds are related directly to the tail vibrations rather than to the direct transmission of sound from the arterial pulsations. In those cases where sound can be heard far beyond the systolic pressure level, it would appear that the sound is due to the impact of blood striking the compressed segment of artery just under the proximal end of the cuff. This would also explain why the latter sound is also synchronous with the pulse. It is not clear why this sound is heard only occasionally and only in the case of certain animals. Its presence might be due to such factors as the location of the caudal artery in relation to the position of the microphone and to the thickness of the tail itself. It would appear that the pulsatile vibrations which can be seen with the naked eye, graphically recorded or amplified in the microphonic manometer into recognizable sound are one and the same. The similar values for systolic pressure by the visual and audiometric methods also support this view. In general, blood pressure measurements made by observation of blood flow after amputating the tip of the tail compared favorably with those made by the other 2 indirect methods when the 20 mm cuff was used. This holds true even for the hypertensive range.

The importance of the tail temperature, cuff width, and cuff position on the simul-

taneous measurement of indirect and direct arterial pressure has been pointed out by Sobin(9) and others(8,10,11). The data in Tables II and III are in agreement with the published observations concerning cuff size.

A serious limitation of indirect blood pressure measurements using the tail occurs when there is severe peripheral vasoconstriction. Thus, when hypertension is associated with vasoconstriction, the tail cannot be used to measure blood pressure by either the visual or audiometric methods. Indeed, the initial heating is necessary in order to overcome the normal vasoconstrictive tone present in the caudal artery. The heating maneuver, however, apparently fails to produce this effect when there is a powerful vasoconstrictor agent, such as aqueous epinephrine, exerting its influence. In the doses used in this study, epinephrine in oil was found to have no such pressor effect. This is in contrast with the findings of Friedman and Freed(7) who reported that 0.2 mg of this drug in oil resulted in doubling the systolic pressure as measured by the microphonic manometer. However, Del Greco *et al.*(8) found that even a much larger dose of this drug dissolved in oil, one milligram, resulted in widely variable blood pressure readings, and they further commented on the reduced tail vibration making it impossible to take readings in some cases.

Summary. A new indirect method for measuring systolic pressure in the rat's tail has been described. It depends upon the visual recognition of pulsatile vibrations which are obliterated by inflating of the cuff to a pressure exceeding the systolic pressure. As the cuff is slowly deflated the vibratory motion reappears, the amplitude of the vibration increasing to a maximum as the cuff pressure approaches zero. The reappearance of the persistent vibration is taken as the systolic endpoint. The accuracy of the method was checked by comparing measurements with values recorded with the microphonic manometer, blood flow after amputating the tip of the tail, and in some instances by direct mean aortic pressure measurements in normal and hypertensive rats. No data were obtained in

hypotensive animals. The advantages and limitations of the visual method have been discussed. The effect of cuff size and preliminary warming of the whole animal or only the tail have been studied.

It is a pleasure to acknowledge the valuable assistance of Dr. Harold Panushka who helped with blood pressure measurements. I am also grateful for technical assistance rendered by Mr. Clifford Nygaard and Paul Masely, and for advice and encouragement of Drs. C. J. Watson, E. B. Flink and Samuel Schwartz.

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An Experimental Aortic Valve. (22949)

EDWARD P. RYAN, GEORGE JOHNSON, JR., AND JOHN M. BEAL
(Introduced by Frank Glenn)

Laboratory for Surgical Research, Department of Surgery, New York Hospital-Cornell Medical Center, New York City.

The correction of aortic valvular insufficiency remains a problem that has not been adequately solved. The insertion of a plastic ball valve by Hufnagel(1) has received the greatest trial, both experimentally and clinically. More recently Silen and his associates (2) attempted to create a competent aortic valve in experimental animals by intussusception of the aorta in its thoracic portion. However, this ingenious method seems to create some degree of constriction. The present report describes experiments in animals, attempting to produce an aortic valve by creation of a flutter valve in the thoracic aorta.

Methods. Mongrel dogs weighing 14-25 kg were anesthetized with nembutal or with ether. Hypothermia (rectal temperature 22-28°C) was induced with an ice bath. Aseptic technics were employed. The left thoracic cage was opened through the fifth intercostal space and the aorta mobilized from the left subclavian artery to the diaphragm. The ad-



FIG. 1. Steps utilized to create aortic valve.

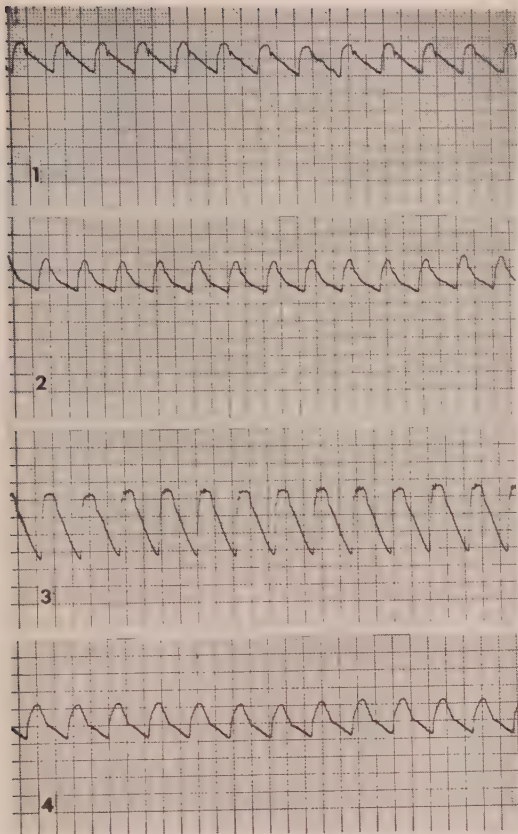


FIG. 2. Tracings of arterial blood pressure were obtained from sixth animal (Table I). 1 is from aortic arch above the newly created valve, 2 was obtained by insertion of catheter through the femoral artery to lower thoracic aorta below the valve. 3 and 4 were taken proximal and distal to the new valve immediately after the cardiac aortic valve had been avulsed.

ventitil covering of the aorta was dissected from a segment approximately 5 cm in length, and Blalock clamps used to occlude the aorta just below the subclavian artery and above the third pair of intercostal vessels. The aorta was occluded for as long as 55 minutes without apparent adverse effects. The intercostal arterial tributaries were controlled by bulldog clamps and the fourth pair of intercostal arteries was divided and ligated. The aorta was transected and the proximal portion bivalved (Fig. 1). Incision along the wall of the aorta was measured to equal the diameter of the vessel. The upper bivalved end was inserted into the lumen of the open distal end and was secured with 4-0 lubri-

cated arterial silk sutures. Occluding clamps were slowly released and the chest closed without drainage. A recovery period of 4 to 7 days was allowed, and left thoracotomy was again performed. Body temperature was not reduced for this procedure. Pressures in the aorta, proximal and distal to the valve, were recorded with Sanborn manometer. In 6 animals the posterior cusp of the cardiac aortic valve was avulsed by a hooked knife through a left ventriculotomy. In these animals pressure recordings were repeated in the same locations following avulsion of the aortic valve. The chest was closed and animals were given digitalis and antibiotics after operation. In 2 animals angiocardiology was performed after creation of the thoracic aortic valve. Intravenous nembutal anesthesia was employed, and 20 cc of 70% Urokon was injected through right jugular vein. One of the animals expired during this procedure.

Results. Four animals died of empyema during postoperative period after pressure recordings had been obtained. Ventricular fibrillation occurred during operation in two. Pressure recordings were obtained in 11 dogs, and in 6 of these, records were made of pressures following avulsion of cardiac aortic valve (Fig. 2). The pressure changes are recorded in Table I. The difference between systolic pressures in the aortic arch and the aorta below the valve varied from zero to 60 mm of mercury, average of 21 mm.

TABLE I. Arterial Pressure Records following Creation of Thoracic Aortic Valve in 11 Dogs.

Aortic arch pressure	Aortic pressure below valve	Systolic pressure differential mm Hg	Aortic pressure after cardiac aortic valve avulsion	
			Aortic arch	Aorta below valve
195/145	160/130	35	145/55	160/130
165/135	105/ 70	60	160/40	105/ 70
130/ 90	100/ 75	30	130/40	100/ 75
150/110	130/110	20	150/45	130/ 85
140/125	140/125	0	140/90	140/ 90
175/105	165/120	10	130/40	130/ 80
185/145	175/135	10		
135/ 85	110/ 70	25		
110/ 80	105/ 55	5		
70/ 40	60/ 40	10		
210/125	185/135	25		

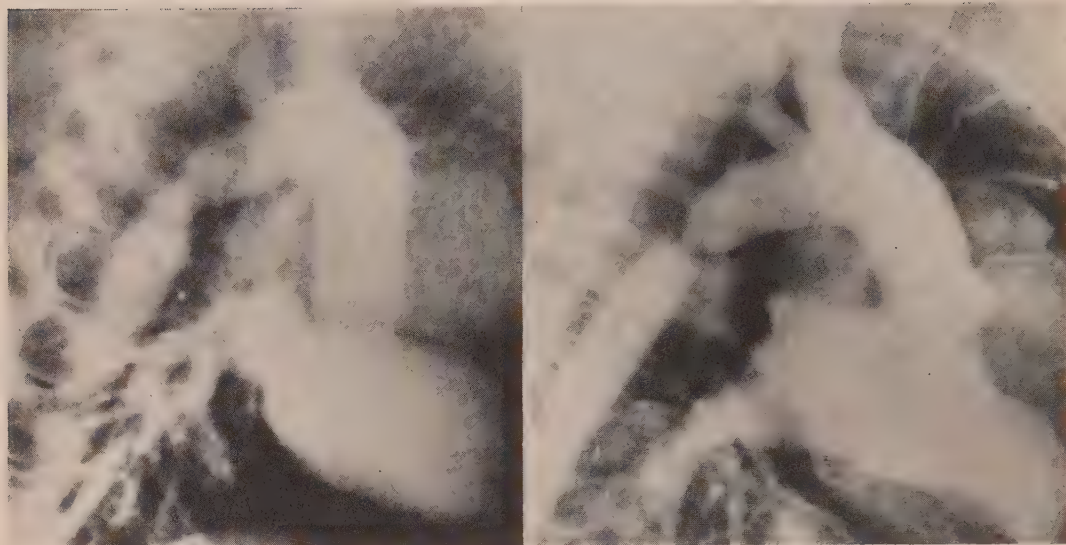


FIG. 3. Aortic phase of the angiograms is presented. Aortic pressure recordings were not determined in these animals.

In the 6 animals in which the aortic valve was avulsed, a satisfactory diastolic pressure was maintained distal to the valve. Elevation of the systolic pressure was not found proximal to the thoracic aortic valve. In each of the 6 animals a marked fall in aortic diastolic pressure was obtained proximal to the valve. In both animals in which angiographic studies were obtained, narrowing at site of creation of the valve was noted (Fig. 3).

Discussion. Construction of a competent aortic valve from autogenous body tissues would be preferable to employment of plastic materials. Plastic materials have the potential danger of creating foreign body reactions, erosion of vessel wall, dislodgement and thrombosis. The method we have described appeared to produce a competent valve. There was little functional evidence of constriction of the lumen of the aorta with the animal at rest. However, in some animals that failed to survive long enough to have pressure recordings made, the valve did not

appear competent and thrombus was present upon the leaflets. A major objection to the current method is the location of the valve distal to the coronary orifices. It is obvious that a longer period of observation is required to determine the effectiveness of this type of valve, particularly in association with avulsion of the cardiac aortic valve.

These preliminary results are believed sufficiently encouraging to warrant further investigation.

Summary. A method has been described for experimental production of an aortic flutter valve. Evidence of competency of the valve has been obtained in 6 animals by means of direct arterial pressure recordings above and below the valve in association with avulsion of the cardiac aortic valve.

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Serum Glyco and Mucoproteins in Rats Treated with Thiourea. (22950)

LUIZ A. ABREU, R. RAPOSO ABREU, AND GILBERTO G. VILLELA
Biochemical Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil.

Abreu, Abreu and Villela(1) found that a significant decrease in serum glycoprotein concentration of thyroidectomized rats was probably related to hypoproteinemia. Lack of reports in the literature on effects of anti-thyroid drugs on serum protein bound carbohydrates led to the present investigation of the effect of thiourea in rat serum glyco and mucoproteins.

Materials and methods. Male Wistar rats weighing 50-60 g were fed *ad libitum* the standard laboratory diet with thiourea added to the milk. The average daily intake of the drug was 20 mg per rat throughout the period of experimentation. A control group of rats was fed the same diet without thiourea. After 60 days of feeding, thiourea treated and control animals were bled by heart puncture under light ether anesthesia and sera separated. Serum glycoproteins and mucoproteins (seromuroid) were determined as protein-bound hexose according to methods described by Winzler(2), and total serum proteins by Weichselbaum's technic(3).

Results. Table I summarizes the data obtained showing average values and standard deviations. The glycoprotein/protein (Gp/p) and mucoprotein/protein (Mp/p) ratios were expressed in mg of glycoprotein or muco-

protein respectively per g of total protein. Table I also includes significances of differences between the averages, calculated according Student's t test(4).

Highly significant inhibition of growth was noted in the thiourea group. However, statistically significant differences in concentrations of glycoproteins, total proteins, and Gp/p relation between normal and thiourea treated rats were not observed. On the other hand, serum mucoprotein levels in this group of animals were significantly lower than those of the control group, and the decrease in Mp/p ratio of goitrogen treated rats was highly significant.

Discussion. Our results obtained with rats fed thiourea have shown that the decrease in seromuroid was not relative but absolute, since the Mp/p ratio was lowered in the absence of significant variations on total serum protein level. This decrease in serum mucoprotein fraction was independent of the total serum glycoprotein concentration, a fact also verified in various conditions(2). However, we found no significant variation in serum glycoproteins and seromuroid in intact rats receiving daily subcutaneous injections of dl-thyroxine or 2,4-dinitrophenol in doses up to 50 μ g or 1 mg per 100 g respectively, during 10 days(5).

Summary. Total proteins, glycoproteins and mucoproteins were determined in sera of normal and thiourea treated rats. Statistically significant decreases were observed only in mucoprotein concentration. Animals fed thiourea showed significantly lower Mp/p ratio without variations in proteinemia and total glycoproteins. Thus the decrease in the mucoprotein fraction was not relative but absolute and independent of total serum glycoprotein level.

TABLE I. Effect of Feeding Thiourea on Serum Glyco- and Mucoproteins in the Rat.

	Controls	Thiourea	P
No. of rats	8	11†	—
Wt (g)	222 $\pm$ 33*	111 $\pm$ 11	<.001
Glycoprotein (mg/100 ml)	117.4 $\pm$ 17.9	104.0 $\pm$ 17.0	<.2
Mucoprotein (mg/100 ml)	9.6 $\pm$ 1.9	6.7 $\pm$ 1.6	<.01
Total protein (g/100 ml)	6.92 $\pm$.48	7.14 $\pm$ 1.24	>.5
Gp/p	16.9 $\pm$ 2.2	14.6 $\pm$ 1.7	<.05
Mp/p	1.4 $\pm$.2	.9 $\pm$.2	<.001

* Mean $\pm$ stand. dev.

† In this group, only 10 animals were used in mucoprotein determinations.

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Cardiovascular Evaluation of a Pressor Amine (Tetrahydrozoline) Showing Hypotensive Properties in Man.*† (22951)

FRANK A. FINNERTY, JR.,‡ JOACHIM H. BUCHHOLZ, AND ROBERT L. GUILLAUME
 (Introduced by John C. Rose)

*Department of Medicine, Georgetown University School of Medicine and D.C. General Hospital,
 Washington, D.C.*

Hutcheon and his colleagues(1) found that in animals, tetrahydrozoline (2-(1,2,3,4-tetrahydro-1-naphthyl) imidazoline hydrochloride) caused an increase in arterial pressure similar to that produced by epinephrine. However, it differed qualitatively from the epinephrine effect in that onset of hypertension was slower and its duration was more prolonged. Increase in blood pressure caused by tetrahydrozoline was diminished, but was never reversed by adrenolytic agents such as phentolamine. The sympathomimetic effect of tetrahydrozoline in animals was so striking that these investigators suggested evaluation of this drug as a pressor agent in certain hypotensive states.

Despite this report(1), preliminary studies in our laboratory indicated that tetrahydrozoline *reduces* blood pressure in man. The present study was undertaken to clarify the hypotensive effect of this sympathomimetic amine.

Methods and materials. The patients in this study were from the medical wards and the hypertensive clinic of the District of Columbia General Hospital. All ward patients who received the drug by injection underwent

complete clinical and laboratory studies. In the clinic, where the patients received tetrahydrozoline by mouth, they were examined at least at weekly intervals and more commonly twice a week for the first month. It was possible to follow 30 of the 42 patients over a 2 to 3 week period on placebo therapy. The hemodynamic studies were carried out on hospitalized patients who were in the fasting basal state. Cardiac output was measured with indicator-dilution curves using Evans Blue dye(2). Serial studies were performed at least 20 minutes apart to assure complete mixing of the dye. Arterial pressure was recorded by the auscultatory method. The average of systolic and diastolic pressures was taken as mean arterial pressure. Total peripheral resistance was calculated by dividing mean arterial pressure by cardiac output (ml/min). Venous pressure was measured using a saline manometer in an antecubital vein at the level of the right auricle.

Results. *Blood pressure changes following tetrahydrozoline given intravenously.* Forty-two patients received tetrahydrozoline intravenously (0.5 mg to 2 mg, average 1.5 mg). In 17 patients, a pressor response was noted (average 35%), beginning less than 30 seconds after injection, reaching its peak within 3 minutes. (Early in this investigation, an immediate pressor response was not specifically looked for, so the actual frequency of this transitory phenomenon cannot be determined accurately. More recent experience indicates that an immediate pressor response consistently follows tetrahydrozoline given intravenously.) A 32% fall in heart rate ac-

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† The authors are grateful for the valuable technical assistance of Miss Erika Bethmann.

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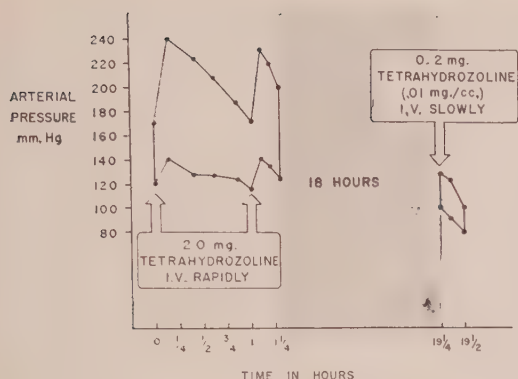


FIG. 1. Chart of arterial pressure of patient G. G. demonstrating response to large and small doses of tetrahydrozoline.

accompanied the hypertensive response in these 17 patients. The pressor period was accompanied by no symptoms.

Fifteen to 20 minutes following administration of parenteral tetrahydrozoline, arterial pressure and heart rate had usually returned to control level. This was followed by a hypotensive phase, which usually began 20 to 30 minutes (average 25 minutes) following initial injection. The peak of hypotensive response was 2.5 to 3.5 hours. Duration ranged between 4 to 12 hours (average 6 hours). During this latter period there was an average fall in systolic pressure of 31 mm Hg (range, 10 to 40 mm Hg). The heart rate decreased an average of 17 beats per minute (range 0 to 50).

Six patients received tetrahydrozoline intramuscularly (0.5 mg to 1 mg). Onset of hypotensive effect in these patients averaged 45 minutes, the peak of action was 2 to 3 hours and duration 6 to 7 hours. Four patients were treated for 5 days. No refractoriness developed during this period.

Differences between large and small parenteral doses were studied as follows: 2 mg tetrahydrozoline were administered rapidly intravenously (Fig. 1). Within 30 seconds, a 24% increase in mean arterial pressure and a 25% decrease in heart rate occurred. In one hour, when arterial pressure had returned to the control level, intravenous injection of 2 mg tetrahydrozoline was again immediately followed by a pressor response. Eighteen hours later, mean arterial pressure was 20%

below the control level. A dose of 0.2 mg (20 ml tetrahydrozoline in concentration of 0.01 mg/ml) administered over a 5 minute period was followed in 15 minutes by further fall in arterial pressure (48% reduction). No pressor phase preceded this hypotensive response.

The reverse of this experiment in another patient gave similar results. Tetrahydrozoline 0.2 mg intravenously was followed in 25 minutes by a 17% fall in arterial mean pressure and a 20% fall in heart rate. In 2 hours, when arterial pressure had returned to control level, repeated intravenous injection of 2 mg of tetrahydrozoline was immediately followed by a 24% increase in arterial mean pressure and 20% decrease in heart rate.

Blood pressure changes following tetrahydrozoline given orally. Tetrahydrozoline was administered orally to 34 moderately severe hypertensive patients. The average effective dosage was 6 to 15 mg/day, given in divided doses. Duration of treatment varied from 4 weeks to 24 weeks (average 14 weeks), except in 2 patients in whom side effects necessitated stopping the drug after 2 weeks. No patient exhibited a hypertensive response. Seven patients had no hypotensive effect either. In 27 patients there was an average fall of systolic pressure of 26 mm Hg (range, 10 to 120 mm Hg). Hypotension was usually accompanied by bradycardia. Although no fall in heart rate was seen in 5 patients, there was an average reduction of 20 beats/minute in 28 patients (range 2 to 46 beats/minute).

A sustained reduction in blood pressure beyond a 10-week period was seen in only 6 patients. Tolerance developed in the remainder of the 21 patients. Once tolerance developed, increasing the dosage to 3 or 4 times the previous level did not cause a hypotensive effect. When the drug was discontinued for a week or 10-day period, a hypotensive effect was again witnessed in the previously effective dosage range.

When placebo therapy was substituted for tetrahydrozoline, a rise in arterial pressure to control level, or higher than control level, was witnessed in each patient.

Cardiac output and total peripheral resis-

TABLE I. Hemodynamic Changes Immediately Following and One Hour after Intravenous Administration of Tetrahydrozoline in 3 Patients.

% change from control to 1 min.				% change from control to 1 hr				
CO	MAP	HR	TPR	CO	MAP	HR	TPR	
-40	+28	-47	+57	-23	-76	-16	-5	
-47	+26	-23	+62	-9	-26	+19	-19	
-22	+41	-42	+55	-51	-40	-11	-8	
Avg	-36	+32	-37	+58	-28	-47	-14	-11

CO = Cardiac output. MAP = Mean arterial pressure. HR = Heart rate. TPR = Total peripheral resistance.

tance. Administration of 0.5 mg to 1 mg of tetrahydrozoline intravenously in 3 patients produced an early and transitory pressor response, as noted above. During this phase there was an increase of 32% in mean arterial pressure, 36% fall in cardiac output, 37% decrease in heart rate, and 58% increase in total peripheral resistance (Table I). One hour from the time of injection of tetrahydrozoline (approximately 40 minutes after the pressor effect) mean arterial pressure had decreased an average of 47%. This reduction in arterial pressure was accompanied by a decrease of 28% in cardiac output and a return of heart rate towards normal (decrease from control value 14%). During the hypotensive phase there was a decrease in total peripheral resistance of 11% below control level.

In 6 subjects receiving tetrahydrozoline orally, a reduction of mean arterial pressure from an average of 171 mm Hg to 143 mm Hg (15%) was accompanied by no significant change in cardiac output and a 20% reduction in total peripheral resistance.

Preliminary studies showed that tetrahydrozoline produced no consistent alteration in the cold pressor response or alteration of the pressor response to adrenalin. Thus, no ganglioplegic or adrenolytic activity was detected.

Bradycardia due to tetrahydrozoline was abolished by administration of 60 to 120 mg of atropine sulfate intravenously in 5 patients, indicating that the bradycrotic effect of the drug was probably mediated by the vagus nerve.

No consistent change in venous pressure

was seen following tetrahydrozoline administration, during either pressor or hypotensive phases. Except for slowing of heart rate and increase of PR interval in 3 patients, no significant changes were found on the electrocardiogram during the pressor or hypotensive phases of the drug.

Discussion. The di-phasic action of tetrahydrozoline on circulation resembles the di-phasic action of epinephrine(3,4). Tetrahydrozoline, like epinephrine appears to possess excitatory and inhibitory properties; the excitatory component causes vasoconstriction and the inhibitory component, decrease of vascular tone. The high initial concentration of the drug causes vasoconstriction; when the excitatory action has worn off, a sufficient concentration of tetrahydrozoline to cause vasodilation may still be present. The pressor effect immediately following the administration of 2 mg of tetrahydrozoline and lack of a pressor response following slow administration of 0.2 mg tetrahydrozoline support this interpretation of the action of the drug (Fig. 1).

According to this concept, parenteral administration of tetrahydrozoline is followed immediately by a hypertensive phase (high concentration in blood) of short duration. The hypotensive phase (minimal blood concentration) continues for that period of time necessary for complete excretion of the drug and therefore is of fairly long duration. Lack of a pressor response following oral administration of tetrahydrozoline can best be explained by lack of complete absorption resulting in low blood concentrations. It is hoped that these studies will stimulate investigation of related drugs for their anti-hypertensive properties.

Summary. 1. Seventeen of 42 patients who received tetrahydrozoline intravenously exhibited an immediate pressor response which lasted an average of 12 minutes. Twenty-five minutes following the initial injection hypotension ensued, lasting an average of 3 hours. The pressor phase was characterized by a decrease in cardiac output, bradycardia and an increase in total peripheral resistance. The hypotensive phase was

characterized by no change in cardiac output, less bradycardia than in the pressor phase, and a decrease in peripheral resistance. 2. When tetrahydrozoline was administered orally, no pressor response was noted. Twenty-seven of 34 patients exhibited an average reduction of 44 mm Hg systolic pressure, 25 mm Hg diastolic pressure, and an average decrease in heart rate of 20 beats per minute. Tolerance usually developed in 7

to 10 weeks.

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Human Liver Succinoxidase.* (22952)

EDWARD E. MASON, YAO WEN LI AND SIDNEY E. ZIFFREN

Department of Surgery, State University of Iowa College of Medicine, Iowa City.

This report deals with conditions necessary for maximum succinoxidase activity in human liver homogenates, the distribution of succinoxidase in subcellular components of human liver and the range of normal for whole homogenates of human liver when "standard conditions" are used(1) and when optimum conditions are used. Waterlow(2), using the Cartesian diver technique, found one-tenth the activity obtained during the present study. Davis *et al.*(3) recently reported succinoxidase Q_{O_2} /mg (dry weight) of slices. The thickness of the slices was not indicated. The incubation medium was not specified beyond saying that Krebs-Ringers succinate was used. A "standard technic" is referred to(1), but this gives only a method for homogenates of small animal tissues and not for human liver slices. How important is it to specify the exact conditions used for measuring succinoxidase activity or to use optimum conditions? What can be considered a normal level of activity? These are questions which led to the following work.

Materials and methods. The selection of patients for biopsy and the preparation of tissue have been described(4). 'Sediment' as

used below indicates all particulate matter in a whole homogenate which is sedimentable at $10,000 \times g$ for 30 minutes with one washing in isotonic sucrose. The reaction mixture used initially was that recommended by Umbreit(1). Standard manometric technics were used with an equilibration period of 10 minutes. Readings were taken for 70 minutes. The reaction was zero order for this period. As the optimum level of each constituent was determined this level was substituted in all subsequent experiments until a final optimum reaction mixture was obtained. Using these optimum conditions, variation of cytochrome c and substrate concentrations were restudied. The pH of the reaction mixtures was determined with a Beckman model G pH meter before and immediately after the experiment was completed. The cytochrome c[†] and sodium succinate were commercial preparations. Ascorbic acid was used for determination of cytochrome oxidase activity. The manometric analyses, including controls for endogenous respiration, were carried out at least in duplicate. The average endogen-

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† Standardization of cytochrome c was spectrophotometrically(1). For an assumed molecular weight of 13,000, various shipments of cytochrome c from Nutritional Biochemicals Co., Cleveland, O., proved to be from 52 to 60% pure. This material gave the same optimum concentration of cytochrome c for rat liver succinoxidase as has been reported(5).

TABLE I. A Fractionation of Human Liver QO_2 (N) Succinate.*

Fraction†	Sedimentation force		Washing	Enzyme activity, μ l/ml, 5% homog./hr	Recovery of enzyme, ‡ %	Total nitrogen, § mg	QO_2 (N)
	× g	Min.					
Whole homogenate				192.20	100	2,755	139.5
Nuclear	211	10	1	14.20	7.39	.587	48.4
I	1,035	10	0	66.35	34.40	.380	349.21
II	1,800	10	"	26.52	13.80	.160	331.5
III	3,200	10	"	23.25	12.10	.115	404.3
IV	5,000	10	"	8.81	4.58	.057	309.1
V	8,890	10	"	16.65	8.62	.048	693.7
VI	18,000	30	"	18.86	9.80	.231	163.2
Supernatant			"	13.77	7.17	1.026	26.84
Total recovery, %					97.86	94.51	

* Distribution of succinoxidase in homogenate of human liver prepared from a biopsy specimen.

† Reaction mixtures contained 1 ml of 0.1M phosphate buffer, pH 7.4, 0.5 ml of 5% whole homogenate or its equivalent of any other fraction, 0.1 ml of 0.5M sodium succinate, 0.1 ml of 12×10^{-2} M $CaCl_2$, 0.1 ml of 12×10^{-2} M $AlCl_3$, 0.9 ml of 2.24×10^{-4} M cytochrome c for the whole homogenate and 0.6 ml of 2.24×10^{-4} M cytochrome c for fractions other than whole homogenate.

‡ Activity of fraction $\times 100$ divided by activity of original homogenate.

§ Per ml of original (10%) homogenate or its equivalent.

ous respiration for all experiments was 4 μ l/hour/0.5 ml of 5% homogenate. Total nitrogen was determined according to Koch and McMeekin(6). The details of fractionation are presented in Table I. Microscopically there was some contamination of the nuclear fraction by mitochondria and the first mitochondrial fraction contained a few nuclei.

Results. Cytochrome c was the moiety investigated first, since it is known that the concentration of cytochrome c in human liver is only about one-fourth that of rat liver(7) and it was anticipated that more exogenous cytochrome c might be required than the 0.133×10^{-4} M recommended for rat liver (1). The whole homogenate of human liver had an increasing succinoxidase activity until the cytochrome c content had been increased to 0.67×10^{-4} M. Approximately 0.45×10^{-4} M cytochrome c is required for maximum activity in sediment (Fig. 1). Cytochrome oxidase was not a limiting factor in these experiments.

The requirement for substrate proved to be lower for human than for rat liver homogenate. The optimum substrate concentration in human liver was the same for both the homogenate and sediment (Fig. 2). An inhibition of activity appeared with increasing

substrate concentration. This same effect was observed by Schneider and Potter(5). Attempts to purify the substrate by reprecipitation had no effect on this inhibition. Since oxygen consumption was observed to be linear with time at all substrate concentrations and since calcium was present in all flasks(8,9), it seems unlikely that an inhibitor such as oxalacetate was formed as a result of higher substrate concentrations. The optimum sub-

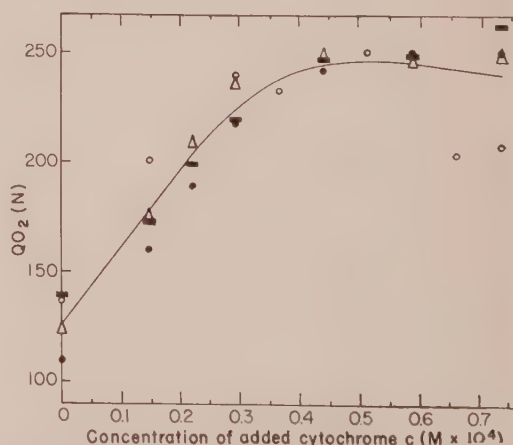


FIG. 1. Relationship of added cytochrome c to succinoxidase activity for sediment using liver biopsies from 4 patients. Sediment includes all particulate matter obtained by centrifugation at $18,000 \times g$ for 30 min and washed once in 0.25 M sucrose.

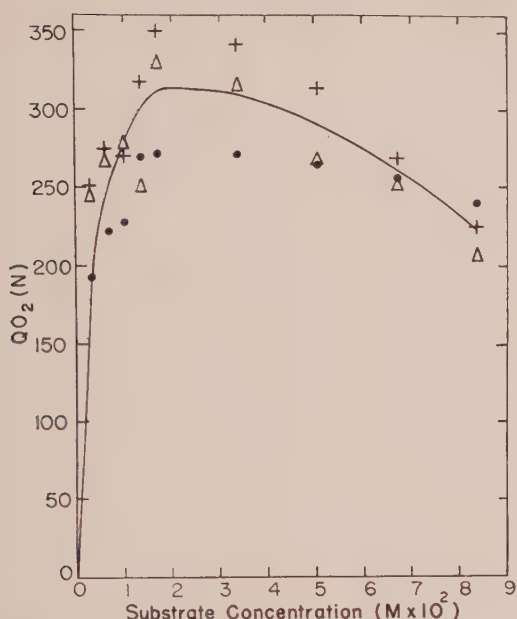


FIG. 2. Effect of substrate on succinoxidase in sediment, 3 exp.

substrate concentration is around $1.6 \times 10^{-2} M$ (Fig. 2). The optimum reported for homogenates of rat liver is $5 \times 10^{-2} M$ (5).

Experiments in which $AlCl_3$ was held constant at $4 \times 10^{-4} M$ and $CaCl_2$ varied, plus experiments with $CaCl_2$ maintained at $4 \times 10^{-4} M$ and $AlCl_3$ varied, revealed optimum activity in all situations where at least 1.2×10^{-3} total cation was present. $FeCl_3$ gave equally satisfactory activity alone or in any combination with $CaCl_2$ and/or $AlCl_3$. pH optimum curves for whole homogenate demonstrated that pH 7.4 is to be recommended for human liver succinoxidase (Fig. 3). Table I shows the results of cell fractionation in a typical experiment. Table II presents data from normal appearing human liver whole homogenates and demonstrates the increase in "normal" succinoxidase activity that results from using optimum conditions for human liver instead of conditions originally worked out for rat liver(1).

Discussion. Succinoxidase in normal adult human liver has an activity of 150 to 200 QO_2/N as determined in whole homogenates under optimum conditions and a QO_2/N of 300 for the sediment from such homogenates (Table II). This is less than one-third the

activity found in rat liver in our laboratory but is considerably higher than the activity that has been reported for normal infant liver using a different technic(2). The known low cytochrome c concentration of human liver plus the requirements for relatively high concentrations of added cytochrome c in homogenized and dilute samples suggests that there may be a more efficient spatial chemical organization in the cells of larger animals. The observations made regarding cation requirements support the thesis that they have a nonspecific effect only. The possibility that calcium is an essential cation in trace amounts is not excluded, however, since no attempt was made to remove the calcium which was already present in the liver. The inhibitory effect of substrate when present in above optimal concentrations has been observed in experiments with rat liver as well but no explanation has been found. The importance of using optimum conditions is illustrated in Table II. As work is done on human tissues repetition will be avoided and the work will be of greater value if conditions are detailed so that they can be reproduced.

Using optimum conditions a number of complete fractionations was carried out as illustrated by a typical experiment in Table I. It was observed that the greatest total concentration of succinoxidase is in the large

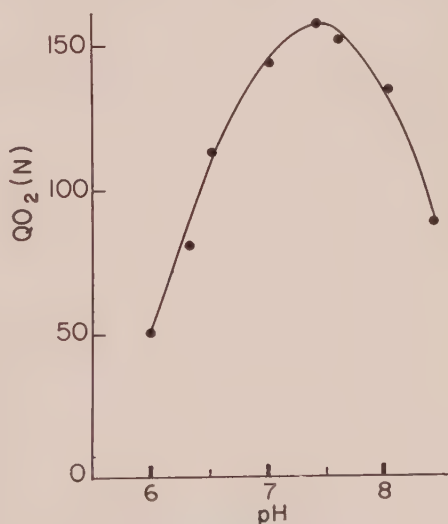


FIG. 3. Effect of pH on succinoxidase activity in whole homogenate.

TABLE II. QO_2 (N) Succinate for a Series of Human Liver Homogenates Using the Standard Manometric Method (Umbreit) as Compared with a Later Series Using Optimum Conditions.

Method	No. of patients studied	QO_2 (N) mean	S.D.†
Conditions recommended for rat liver(1)	6	76.4	8.8
Optimum conditions for human liver*	10	177.2	30.2

* See footnote to Table I.

$$\dagger \text{Stand. dev.} = \sqrt{\frac{\sum (x^2)}{N}}.$$

granule fraction. The activity seen in the nuclear fraction can be explained by contamination with large mitochondria and likewise the small particles of fraction VI contain some mitochondrial material as well as most of the microsomes. The distribution of succinoxidase in subcellular particulate matter was observed to be similar in human liver to the distribution in rat liver and illustrates again the heterogeneity of mitochondria(10, 11). Succinoxidase is a better enzymatic identification of mitochondrial material in homogenates of human liver than fumarase, citrase or isocitrase(4).

Summary. The range of succinoxydase activity in whole homogenates of human liver is 150 to 200 QO_2 /N. The requirements for added cytochrome c are high in spite of a known low concentration of endogenous cytochrome c, suggesting a more efficient spatial chemical organization within the intact cells of larger animals. The distribution of suc-

cinoxidase in subcellular particles is shown. The requirements for optimum succinoxidase activity are indicated and it is demonstrated that the activity is more than doubled when optimum concentrations of flask contents are used as compared with concentrations originally developed for small animal tissue homogenates. Succinoxidase is a good enzymatic identification for mitochondria in studies on the chemistry and enzymatic activity of human liver cell fractions.

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Relationship Between Onset of Egg Production and Plasma Cholesterol Levels in the Chicken.* (22953)

G. LEVEILLE, HANS FISHER AND H. S. WEISS. (Introduced by Gerald Litwack)

Poultry Department, Rutgers University, State University of New Jersey, New Brunswick.

With the current interest in plasma cholesterol level and its relationship to atherogenesis, it seemed of interest to report the pattern of plasma cholesterol during maturation in the chicken. Lorenz *et al.*(1) observed a rise in the level of blood cholesterol and total lipids during puberty. The present study extends these observations and demonstrates the biphasic nature of this rise as related to the onset of egg production.

Method. Plasma cholesterol was determined in a group of 20 Leghorn pullets at 3-4 week intervals beginning at 12 weeks of age. All birds were maintained in individual cages in a temperature regulated room. Cholesterol was determined by the method of Zlatkis *et al.*(2).

Results. The pattern of plasma cholesterol with increasing age is shown in Table I. Plasma cholesterol was observed to rise significantly ($P < 0.01$) from an initial value of 116 mg % to a value of 285 mg% at 168 days of age, which represents an increase of 146% over the initial value. Following this peak, there occurred a statistically significant ($P = 0.01$) decrease to 208 mg% which, however,

still represents a significant 79% increase over the initial value. The occurrence of the peak cholesterol level corresponds in time very closely to the onset of egg production; the average number of days to deposition of the initial egg was 155 days.

Discussion. The observed increase in the level of plasma cholesterol which confirms a previous report(1) may be due to the increase in estrogen activity at the time the bird begins laying. It must also be borne in mind that fat biosynthesis from the all-carbohydrate caloric intake of the bird plays only a minor role in the intermediary metabolism of the pullet prior to the commencement of egg production. The significant decrease from the peak value undoubtedly represents a physiological adjustment by the bird to the new stress of egg production and the concomitant deposition of large amounts of fat in the yolk. Unpublished data from this laboratory suggest that a similar initial rise and subsequent decline in plasma cholesterol may occur with continued administration of estrogen or cholesterol to mature hens.

Since the chicken has become a widely used experimental animal in the study of sclerosis and cholesterol metabolism, the present study points up the importance of ascertaining the stage of development of experimental birds. If females are to be used, it would seem desirable to select for experimental purposes either non-laying immature birds or females in active production for several months.

Summary. Plasma cholesterol levels have been studied as a function of age and onset of egg production in Leghorn pullets. Following a highly significant increase of 146% with peak value corresponding closely to time of first egg, plasma cholesterol decreases significantly from peak value to 79% above initial value. It is suggested that this relationship be considered when choosing birds for

TABLE I. Relationship of Plasma Cholesterol Level to Onset of Egg Production.

Age of birds (days)	% of birds in egg production	Plasma cholesterol (mg %)
84	0	116 $\pm$ 2*
105	0	152 $\pm$ 2
133	5	223 $\pm$ 31
168	90	285 $\pm$ 24
172	100	
198	100	208 $\pm$ 15
232	100	238 $\pm$ 16

* Stand. error of mean.

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Phenaglycodol:* A New Antiepileptic Agent. (22954)

CHARLES M. GRUBER, JR. AND JACK M. MOSIER.†

(With assistance of Charles Lofton†)

Lilly Laboratory for Clinical Research, Indianapolis General Hospital, Indianapolis, Ind.

Phenaglycodol (2-p-chlorophenyl-3-methyl-2, 3-butanediol) has, by rat screening methods, anticonvulsant and sedative properties.‡ These authors report the following oral doses for rats: electroshock $ED_{50} \pm SE = 63.0 \pm 6.6$ mg/kg; metrazol $ED_{50} \pm SE = 45.1 \pm 7.7$ mg/kg, ataxia $ED_{50} \pm SE = 86.5 \pm 6.1$; hypnotic $ED_{50} \pm SE = 165.7 \pm 16.6$ mg/kg; and the $LD_{50} \pm SE = 832.4 \pm 37.5$. Evaluation in humans with difficult to control grand mal, petit mal or mixed epilepsy also indicates anticonvulsant activity.§ An attempt was, therefore, made to demonstrate anticonvulsant activity in patients with focal brain damage and epilepsy.

Method. Twelve patients with focal brain damage and epilepsy received identically appearing experimental medications orally every 8 hours. Each medication was given for 3 days of one week. The medications were randomized among the patients. Focal and generalized seizures were reported by attendants who had no knowledge of the medications given. Each focal seizure was scored

1 point and each generalized seizure was scored 2 points to give a daily seizure score for each patient. The score on the fourth day when their regular medications were again given was also included. Patients with no seizures during the study periods were excluded as non-epileptics; patients with more than 6 seizure points during any 8-hour period were given sodium phenobarbital intravenously and excluded from the study. A detailed description of this method has been reported previously(1).

Results. The results with the various medications are presented in Table I. As variance analysis (Table II) indicates, significant ($p > 0.05$) effects were produced by both phenaglycodol and phenobarbital. The two together apparently have an additive effect.

Discussion. Phenaglycodol lacks the structural characteristics which have been associated with anticonvulsant activity. In comparison with phenobarbital, larger doses are required; however larger doses are also tol-

TABLE I. Sums of Daily Seizure Scores from 12 Patients. Totals are given in parentheses.

	Phenobarbital in mg												Total			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Phenaglycodol, 400 mg	8	6	17	5	6	11	13	9	0	4	2	4	14	21	32	18
			(36)				(39)					(10)			(85)	
Blank	8	18	51	12	2	14	26	8	2	6	6	4	12	38	83	24
			(89)				(50)					(18)			(157)	
Total	16	24	68	17	8	25	39	17	2	10	8	8	26	59	115	42
			(125)				(89)					(28)			(242)	

* 'Ultrán' (Phenaglycodol, Lilly).

† New Castle State Hospital, New Castle, Ind.

‡ To be published.

§ Personal communication.

TABLE II. Analysis of Data Presented in Table I.

Source	Sum of squares	Degrees of freedom	Mean square
Patients	85	11	7.7
Phenaglycodol	18	1	18.0*
Phenobarbital,	50	2	25.0*
25 mg vs 100		(1)	19.4*
mg blank		(1)	30.6*
Days	63	3	21.0*
Remainder	1,176	270	4.35
Total	1,392	287	

* Significant at $p > 0.05$.

erated by humans without excessive central nervous system depression. Comparable

doses can therefore be given.

Usually drugs effective in grand mal are expected to increase the severity of petit mal and vice versa. Since phenaglycodol is effective in petit mal and the generalized seizures occurring in patients with focal brain damage, it may prove advantageous in epilepsy of the mixed type.

Conclusions. Phenaglycodol (Ultran, Lilly) is an effective anticonvulsant in epilepsy associated with focal brain damage.

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Protection of Mice with Polyvinylpyrrolidone Against Lethal Doses of Tetanus Toxin. (22955)

HEINZ BICK, ROBERT S. ABERNATHY, AND WESLEY W. SPINK

Department of Medicine, University of Minnesota Medical School, Minneapolis, Minn.

The polymer polyvinylpyrrolidone (PVP) was introduced as a plasma expander by Hecht and Weese(1). Other German investigators(2,3) subsequently demonstrated that in experimental animals, injections of PVP caused urinary excretion of various dyes normally retained in the body. Stern(4) showed later that in mice PVP enhanced renal excretion of dyes, prevented storage of dyes in the reticuloendothelial system, and also displaced dyes already stored in the reticuloendothelial system. Prompted by these results, Schubert (5) and later Krech(6) described in both experimental animals and human cases a beneficial action of PVP against exotoxins of tetanus, diphtheria, and botulism. Further experience of the German workers(7-11) indicated that PVP with a molecular weight of 12,600 (Periston N or PVPN) was more effective, presumably because it was more rapidly and completely eliminated in urine than the original PVP having a molecular weight of 30,000.

Our previous study demonstrated that PVPN protected ABC mice against lethality of brucella endotoxin(12). In experiments

summarized here, the first objective was to confirm the enhancing effect of PVPN upon urinary excretion of trypan blue in animals. Following this demonstration, the action of PVPN upon lethality of tetanus toxin was investigated and compared to its effect on trypan blue. As will be shown, the action of PVPN upon these two agents differed significantly.

Material and methods. In investigations relating to excretion of trypan blue, male albino rabbits were selected for use, since accurate quantitative collections of urine could easily be obtained by urethral catheterization. At various intervals following intravenous injection of trypan blue (13 mg/kg body weight), PVPN* in an amount approximating 20% of estimated blood volume was given intravenously. Urine was collected by urethral catheterization both prior to and following injection of PVPN. The amount of dye in the urine was determined colorimetrically, using as standards known amounts of trypan blue

* Obtained from Die Farbenwerke Bayer, Leverkusen, Germany.

TABLE I. Effect of PVPN on Urinary Excretion of Trypan Blue in Rabbits.

Treatment	Time of PVPN admin., hr	No. of animals	% total dose trypan blue excreted after Periston-N	
			2 hr	4 hr
Saline		7	0	0
PVPN	S*	10	22 (15-33)	24 (16-36)
"	24	3	9 (8-12)	12 (11-12)
"	48	1	12	13
"	72	1	4	4

* S = Simultaneous.

dissolved in normal rabbits' urine. The amount of trypan blue excreted in urine was then expressed as per cent of total dose injected. Male ABC mice, averaging 20 g in weight, were used in experiments with tetanus toxin. All injections were made either intravenously through tail vein or intraperitoneally. The tetanus toxin† was stored at 4°C until use. Challenge was made with 0.1 ml of various dilutions, which were always freshly prepared to avoid spontaneous conversion to toxoid. Further treatment consisted of 0.5 ml of PVPN (6% solution) or 0.5 ml of sterile saline. When the toxin and PVPN were given simultaneously, they were mixed and injected from the same syringe. All other injections were given from separate syringes.

Results. Effect of PVPN on Excretion of Trypan Blue. Injection of trypan blue gave immediate bluing of skin and sclerae in control rabbits. No appreciable excretion of the dye in urine was apparent over a 72 hour period. When PVPN was given simultaneously with trypan blue, the degree of tissue staining was often less than in controls, but this was not consistent. The most striking and consistent effect of PVPN was to cause excretion of trypan blue in urine (Table I). Maximal excretion of the dye occurred within 2 hours; a small additional amount was found at 4 hours but none thereafter. When PVPN was given simultaneously with the dye, all rabbits tested promptly excreted grossly blue urine containing a considerable portion of the dye. Animals receiving PVPN 24 or 48 hours after trypan blue also had blue urine, but

excreted less of the dye. Even at 72 hours a slight but definite excretion of trypan blue occurred in one rabbit.

Effect of PVPN on Lethality of Tetanus Toxin. Two lots of tetanus toxin were used. Both produced typical tetanic muscular spasms and generalized convulsions when given either intravenously or intraperitoneally. Since the majority of studies were made with lot #29577, a detailed titration of its potency is presented in Table II.

The effects of injections of PVPN upon lethality of tetanus toxin are shown in Table III. When tetanus toxin and PVPN were given intravenously and simultaneously (Exp. 1), excellent protection resulted. However, if therapy with PVPN was delayed for 24 hours (Exp. 2), no protection occurred. When toxin and PVPN were given at the same time intraperitoneally (Exp. 3), the only significant protection was against the minimal lethal dilution of 1:65,000. In Exp. 4, the effects of varying the timing, the number, and route of injections of PVPN were evaluated against toxin administered intraperitoneally. Neither multiple intraperitoneal injections nor intravenous injections afforded any protection against this challenge.

To confirm these results, the efficacy of PVPN was tested against a second lot of tetanus toxin (#29495). This was found to be less potent than lot #29577, necessitating injections of more concentrated dilutions to obtain lethality. As shown in Exp. 5, Table III, protection with PVPN was found only when toxin and PVPN were given intravenously.

Discussion. Studies with trypan blue con-

TABLE II. Lethality of Tetanus Toxin (Lot #29577) in ABC Mice.

Dilution of toxin (× 1000)	Intrav.		Intraper.	
	Deaths*	Avg day of death	Deaths*	Avg day of death
1: 5	5/5	1	5/5	1
1: 20	5/5	2	5/5	2
1: 40	2/2	3	5/5	2
1: 50	0		5/5	2
1: 60	5/5	3	4/5	4
1: 70			0/5	—
1: 80	4/5	3	0/5	—
1:100	7/7	4		

* No. dying/No. challenged.

† Obtained from Merck-Sharp & Dohme, West Point, Pa.

TABLE III. Effect of PVPN on Lethality of Tetanus Toxin in Mice.

Exp.	Dilution of toxin ($\times 1000$)	Route* of toxin	Route* of PVPN	Timing of PVPN (hr)	Results of treatment			
					PVPN		Saline	
					Death†	Avg day of death	Death†	Avg day of death
1	1:60	I.V.	I.V.	S†	0/20		15/15	5
	1:80	"	"	S	2/20	7	10/10	5
2	1:80	"	"	S	2/10	7	"	4
	"	"	"	24	10/10	4		
3	1:50	I.P.	I.P.	S	20/20	2	15/15	2
	1:55	"	"	S	7/10	7	10/10	3
	1:60	"	"	S	8/10	4	12/15	4
	1:65	"	"	S	2/10	7	10/10	3
4	1:50	"	"	S	9/10	4	15/15	2
	"	"	"	24	10/10	2		
	"	"	"	24 + 48	9/10	3		
	"	"	"	S + 24 + 48	10/10	3		
	"	"	I.V.	S	"	2		
	"	"	"	24	"	2		
5	1: 2	I.V.	"	S	0/35		27/35	6
	"	I.P.	I.P.	S	40/41	4	31/41	4
	1: 4	"	"	S	20/20	6	18/20	7

* I.V. = Intrav.; I.P. = Intraper.

† No. dying/No. challenged.

‡ S = Simultaneous.

firm the observations by Schubert(2,5) and Stern(4) that polyvinylpyrrolidone has a "washing out" effect upon dye stored in tissues. In contrast, however, such an action upon tetanus toxin was not observed. Although distinct protection with PVPN was demonstrated, this occurred only under restricted experimental conditions in which both toxin and PVPN were given intravenously and simultaneously. Lack of protection when therapy with PVPN was delayed has also been noted by Krech(6). This observation suggests that PVPN has no effect on tetanus toxin which has already been fixed by the tissues. Such an explanation has also been proposed by Benchelt.‡ The ineffectiveness of PVPN against toxin given intraperitoneally may result in a similar fashion. The explanation of the discrepancy between actions of PVPN on stored trypan blue and on fixed tetanus toxin is not clear but may reflect either a greater affinity of tissues for tetanus toxin than for trypan blue or an alteration in the chemical or physical properties of the toxin molecule by tissues so that PVPN can no longer combine with it.

These experiments point out the limited magnitude of the protective action of PVPN

against tetanus toxin. This would suggest that therapy of human tetanus with PVPN would be of limited value, since only rarely can patients be treated simultaneously with liberation of toxin in the body. In support of this is the observation that PVPN had no beneficial effect in 10 patients with severe tetanus(13).

Summary. Intravenous injection of polyvinylpyrrolidone (PVPN-molecular weight 12,600) caused urinary excretion of trypan blue in rabbits, even when given 48 hours following injection of the dye. PVPN also protected mice against tetanus toxin but only when both toxin and PVPN were given intravenously at the same time. Since no protection was observed when therapy with PVPN was delayed or when the toxin was given intraperitoneally, it was suggested that PVPN was ineffective against tetanus toxin fixed in the tissues.

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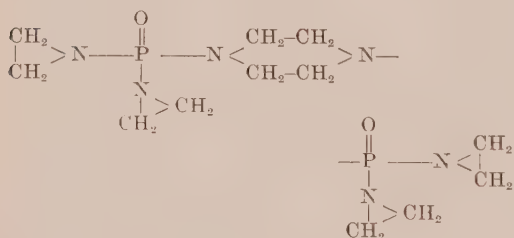
IV. Chemotherapy of Tumors in Rats with Piperazine-1,4-bis (N,N'-diethylenephosphondiamide).^{*} (22956)

M. L. CROSSLEY, J. B. ALLISON, D. R. SEEGER AND E. KUH

Bureau of Biological Research, Rutgers University, New Brunswick, N. J., and Calco Chemical Division, Am. Cyanamid Co., Bound Brook, N. J.

This investigation is a continuation of the study of ethylenephosphoramides in the treatment of transplantable tumors in rats (1).

Materials and methods. Piperazine-1,4-bis (N,N' - diethylenephosphondiamide), (PDA) is a white crystalline compound; m.p. 185-186°C: very soluble in water and alcohol but almost insoluble in acetone and ether. It is slightly hygroscopic. Its structural formula is:



It has been reported to be active against a granulocytic chloroleukemia of the rat.[†] Adult, male Sprague-Dawley and Wistar rats, averaging about 150 g were used. In each experiment there were, as a rule, normal control (NC), tumor control (TC), drug control (DC), and tumor treated (TT) groups

consisting of 8-10 animals each. The number of groups varied with dose levels of the drug chosen for comparison in any one experiment. An isotonic saline solution of the drug prepared weekly and kept at about 5°C, was given in one daily dose by intraperitoneal injection, the volume being that required to provide the desired dose in mg/kg of body weight of each rat at time of treatment. The Flexner-Jobling carcinoma and the Walker 256 carcinosarcoma were studied in Sprague-Dawley rats, the sarcoma R1 in Wistar rats. Methods of implantation and measurement of growth were essentially the same as those previously described (2). The animals were weighed periodically, length and width of the tumors measured in millimeters and size expressed as mm². Therapeutic effectiveness was determined by comparing average size of tumors of treated groups with that of untreated controls. When possible tumor weights were also compared. Regression was considered complete when the tumor was no longer palpable, no regrowth occurred in about 2 weeks, and gross examination at autopsy showed no evidence of tumors. Treatment of rats with Flexner-Jobling and R1 tumors began 5 days after implantation and with Walker Carcinosarcoma 256 3 days. The rats were group pair-fed, using 18%

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[†] Sparks, S. *et al.* abstract published.

TABLE I. Effect of PDA on Sprague-Dawley Rats, with and without Flexner-Jobling Tumor.

Rat group*	Dose, mg/kg/D	Days after implantation	Days treated	% carcass gain	g tumor/100 g carcass wt	% regressions	% survival	g organs/100 g body wt	
								Spleen	Testes
NC		22		54			100		1.32
TC		26		48	14.0	0	90	1.20	1.07
DC	.2		22	50			100		1.37
TT	.2	40	35	72	12.5	30	100	.40	.77
NC		58		105			100	.19	.95
TC		34		66	20.5	0	90	.97	1.00
DC	.5		53	104			100	.31	.74
DC	1.0	58	53	105			80	.16	.54
TT	.5	58	53	109	3.0	30	85	.32	.79
TT	1.0	58	53	107	0.7	30	90	.34	.58
NC		29		31			100	.26	
TC		29		39	12.0	0	80	.80	
TT	3.0	29	24	25	0.3	90	100		
TT	3.0	43	38	56	0.2	90	90	.33	.56
NC		36		86			100	.21	1.23
TC		36		50	12.0	0	70	.67	1.17
DC	3.5	36	31	76			80	.18	.59
TT	3.5	36	31	61	1.0	85	70	.35	

* NC, normal controls; TC, tumor controls; DC, drug controls; TT, tumor treated.

casein diet(3). The duration of the experiments was usually from 4-6 weeks. Blood counts were taken for certain groups, particularly the normal and the drug controls, before treatment began and again just before it ended. This and other indications of toxicity, such as carcass weight change, development of anemia, and general well being of the animals were recorded. Organ weights were expressed in g/100 g of gross body weight. Weight of tumor is given in g/100 g of carcass weight. All the data are from group averages.

Results. Sprague-Dawley rats given doses of 0.2 to 1.0 mg/kg/D showed no significant leukopenia or other toxic symptoms after 20-30 days of continuous treatment. After 53 days treatment, there was a significant drop in testes weight of 30-40%. With doses of 2.0-4.5 mg/kg/D there was a drop in white blood cell counts after 30-40 days of about 40% but it was no greater for the larger dose than for the smaller.

Animals bearing the Flexner-Jobling carcinoma were treated with doses of .2, .5, 1.0, 3. and 3.5 mg/kg/D for periods of 24-53 days. The tumor regressed completely in about 30% of the animals receiving the 0.2 mg dose after about 6 weeks. At this time another 20% had very small tumors, averaging less than 0.5 g/100 g carcass weight.

In the remaining 50%, tumor growth seemed to be unrestrained by the drug. The tumor averaged 25 g/100 g carcass weight after 35 days of treatment (DT) or 40 days after implantation (DaI). The tumors were affected in 100% of the animals treated with 0.5 and 1.0 mg/kg/D for 53 days; 30% regressed completely; the remaining 70% regressed to a very small size averaging 3 g and 0.7 g/100 g carcass weight respectively. Occasionally this tumor in the Sprague-Dawley rat will regress spontaneously in a limited number of cases. Under the conditions of this experiment there were no regressions among the tumor controls in 34 days when the tumors were large, averaging 21 g/100 g carcass weight. With a dose of 3 mg/kg/D the tumors regressed completely in about 90% of the animals, the remainder diminishing to very small size. There was little difference in the results when treatment was prolonged from 24-38 days. Similar results were obtained with 3.5 mg/kg/D for 31 days. This piperazine compound has a wide therapeutic range which makes it interesting for further investigation.

The R1 sarcoma was retarded in growth by doses of 1.5 and 3.0 mg/kg/D but none of the tumors regressed. Treated animals lived longer and their carcass weight gain was greater than that of the tumor controls. In

2 weeks the results of treatment were:

Group	Dose, mg/kg/D	g tumor per 100 g carcass wt	% carcass gain
TC		20	3
TT	1.5	14	15
TT	3.0	9	27

A dose of 0.5 mg/kg/D did not affect growth of the Walker carcinosarcoma and 1 mg/kg/D caused only slight retardation in tumor growth. With a dose of 3 mg/kg/D, tumor growth was considerably retarded and survival time of the experimental animals lengthened. In 24 days of treatment, the tumor averaged 5 g/100 g carcass weight, in comparison with 42 g for the TC group 15 days after implantation. Treatment with a dose of 4.5 mg/kg/D inhibited growth of tumors for a time, but after 2-3 weeks growth resumed and continued at a rapid rate in spite of daily therapy. Ultimately tumors in treated rats grew to be as large as those in the controls, which, however, died in much shorter time. The tumor became resistant to the drug. In becoming resistant it seemed to have changed its character permanently and its growth was not thereafter inhibited by the same dose, even after 5 serial transplantations into new hosts. The tumor which had become resistant to the 4.5 mg/kg/D dose was taken for serial transplantation just after growth resumed and when it was still small. The treatment each time was with a daily dose of 4.5 mg/kg. Treated animals were the donors of the tissue for the next transplant. Growth of the tumor in treated rats was retarded each time in comparison with that in untreated, but not inhibited. It has been previously reported that the Walker carcinosarcoma developed resistance to TEPA and MEPA(4).

In these experiments the treatment had been continuous, *i.e.*, daily and with the same dose of drug throughout. It was desirable to know if this was necessary and if there was any difference in resistance to the drug when it was administered for a shorter time. It was also of interest to know if regression of growth was influenced by following the early treatment of a large dose, with a small main-

TABLE II. Tumor Size, mm² (W-256).

Days treated	I	II	III	IV
3	142	182	107	193
5	187	148	110	551
7	191	144	129	1480
12	273	252	170	2853
17	316	334	192	5368
21	342	427	247	
24	354	469	331	
27	785	987	456	

tenance dose of either the same drug or another of the same series. Forty Sprague-Dawley rats were implanted with the Walker tumor and divided into 4 groups of 10 rats each. Group I was treated with 4.5 mg/kg/D of PDA for 4 weeks. Group II received treatment for 2 weeks. Group III was treated with the same dose for 2 weeks, then the drug was changed to TSPA, in doses of .4 mg/kg/D for 2 weeks. Group IV was untreated. The effect on growth of the tumor was as shown in Table II.

The period of growth inhibition was about the same in the 3 cases. The tumors became resistant in all 3 treated groups. It was possible that the processes of resistance had already set in during the 2 weeks treatment with the 4.5 mg/kg/D dose. Treatment with 4.5 mg/kg/D for 1 week, followed by maintenance dose of 1 mg/kg/D for 3 weeks did not prove to be adequate for growth inhibition.

With a dose large enough, growth of this tumor was inhibited when treatment was started 3 days after implantation. An important question was the behavior of the tumor when treatment was delayed. With tumors permitted to grow to a size about 500 mm², before treatment began, a daily dose of 4.5 mg/kg/D, inhibited growth for a few days but later resistance developed.

Summary. 1. Piperazine-1, 4-*bis*(N,N'-diethylenephosphondiamide) (PDA) in doses up to 1 mg/kg/D for 4-6 weeks produced no significant toxic reaction in rats; at higher doses there was some leukopenia but it was not serious, even with doses of 3.0-4.5 mg/kg/D administered for 4-6 weeks. 2. Flexner-Jobling carcinoma regressed completely in 30% of rats treated with .2 mg/kg/D of PDA and in about 90% of those with 3.5 mg/

kg/D. There were no regressions among the controls 34 days after implantation. 3. Growth of sarcoma R1 in Wistar rats was retarded about 50% by the product at 3 mg/kg/D. 4. Walker 256 carcinosarcoma was retarded in growth by doses of 1-3 mg/kg/D and inhibited for about 2-3 weeks with a dose of 4.5 mg/kg/D. 5. After initial period of growth inhibition the Walker tumor became resistant to the drug and then grew rapidly in spite of continued therapy.

We acknowledge with deep gratitude our indebted-

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Effect of Preweanling Administration of Sucrose on Subsequent Caries.* (22957)

RALPH R. STEINMAN AND MELVIN I. HALEY. (Introduced by U. D. Register)
*School of Dentistry and Department of Biochemistry, College of Medical Evangelists,
Loma Linda, Calif.*

Sognnaes(1,2) indicated that ingestion of sucrose by dams of rats and monkeys during the calcification of teeth of the young may affect their subsequent caries experience. In another study(3) ashed laboratory chow, fed to pregnant rats, decreased the amount of decay in the offspring. This observation might suggest that the increased caries, using the refined diet alone, was due to lack of some important constituent in the diet. The technic developed by the authors[†] in which various food components were given directly to the young during the suckling period was very useful in studying the systemic effect of diet in caries. When using this technic various carbohydrates given to the suckling young in doses of approximately 1 to 1000 by body weight produced a marked increase in decay of teeth undergoing calcification. Of the carbohydrates used from the 2nd day following birth to weaning, sucrose produced the most consistent increase in caries. The present study was designed to show the time dur-

ing suckling period that these changes take place.

Method. Thirty-six new born caries-susceptible rats from 6 litters were divided into 4 equal groups. Twenty percent sucrose solutions were administered orally, using amounts not greater than 1 to 1000 of body weight. One-fourth of this group received drops 3 times a day from the day following birth through the 6th day. One-fourth received drops from the 7th through the 13th day. The next group received drops 3 times a day from 14 through the 21st day. The last 4th received only maternal milk throughout the suckling period. All animals received mothers milk *ad libitum* during the suckling period. When weaned at 21 days of age all animals were placed on the cariogenic diet of the following composition: Casein 24, sucrose 65, salts U.S.P. XIV 4, corn oil 5, dehydrated whole liver 4, vitamin mix(4) 2, choline 0.2 parts.

Animals were sacrificed at the end of 13 weeks and the molars examined by grinding successive planes parallel to the occlusal surface. Individual charts which showed the relationship of the enamel, dentin, and the pulp

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[†] Reported at Internat. Assn. for Dental Research, 1956 meeting.

TABLE I. Time of Administration of 20% Sucrose and Resulting Caries Score 13 Weeks Later.

Time, days	1st molar	2nd molar	3rd molar	Total	P value compared with control
1 to 6	20.4	13.6	3.7	37.7	<.01
7 to 13	22.7	15.8	3.3	41.8	<.03
14 to 21	23.2	17.7	5.4	46.3	<.02
Control*	11.1	7.1	1.6	19.8	

* Nine animals/group.

of each molar were used to record the carious lesions. These lesions were scored by the method of Shaw(5). Table I shows these results.

Results. The results of this study are in agreement with the previous work in which a marked increase in the decay was found following administration of various carbohydrates by this technic.

In this study over 100% increase in decay of the teeth was found in the 1st, 2nd, and 3rd molars over litter mates which received only maternal milk during the suckling period. There appears to be no significant difference as to which 3rd of the suckling period the sucrose is given. The 1st molar of the albino rat begins to calcify at about birth and erupts at the 19th day. The 2nd molar begins to calcify about the 2nd day following birth and erupts at about the 22nd day. The 3rd molar begins to calcify about the 15th day and erupts at about the 35th day following birth(6). The differences in decay in

this study between the animals receiving the 20% sucrose solution and those receiving maternal milk only would indicate that these changes occur throughout the period of calcification. When it is considered that the changes were induced by giving the sucrose solution for only 7 days, it would appear that these changes are systemic in nature and that they are irreversible. The earlier part of the suckling period has fewer variables and for this reason is being used in further studies of food components and their effect upon the calcifying teeth.

Summary. An effective technic has been found to alter tooth structure which results in an increase in decay of these teeth. This technic specifically separates the systemic from the purely local factors in the development of caries. Twenty % solution of sucrose given orally 3 times a day for at least 7 days during the calcifying period resulted in over 100% increase in decay of teeth of white rats over litter mates not so treated.

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Effect of pH on Cytopathogenicity of Orphan Viruses.* (22958)

ALMEN L. BARRON AND DAVID T. KARZON.† (Introduced by Ernest Witebsky)

Departments of Bacteriology and Immunology, and Pediatrics, University of Buffalo School of Medicine, and Statler Research Laboratories of Children's Hospital, Buffalo, N. Y.

Orphan viruses represent the third virus group, in addition to poliovirus and Coxsackie virus, isolated from human enteric tract and associated with poliomyelitis-like disease(1). Fourteen serotypes of orphan viruses have been classified as ECHO (enteric cytopathogenic human orphan) viruses(2,3). The role of ECHO viruses in human illness, with special reference to aseptic meningitis (non-paralytic poliomyelitis), was recently discussed(4,5). During the course of an investigation of an outbreak of seasonal aseptic meningitis in western New York in 1955, it was found that the cytopathogenic effect of certain orphan viruses in monkey kidney tissue culture was depressed in the presence of an acid pH in tissue culture fluid.

At the present time, the sole method of isolation and titration of orphan viruses is the detection of their cytopathogenic effect in simian or human tissue cultures. It appeared important therefore to investigate the effects of pH change upon sensitivity of the system. Some of the factors influencing this phenomenon are reported in the present study.

Materials and methods. Tissue culture system and media. Monkey kidney trypsin-dispersed monolayer tissue cultures were used (1). Two nutritionally similar media were employed, one with a low and the other with a high buffer capacity. The low buffer medium consisted of 0.5% lactalbumin hydrolysate, 2% calf serum in a base of Hanks' balanced salt solution(6,7). The high buffer medium substituted Earle's saline(7,8) for the Hanks' balanced salt solution. The pH was determined colorimetrically, using phenol red indicator in the medium, by comparison with color standards. The pH of both media

TABLE I. Composition of Buffer Systems of Media.

Constituent	Concentration/100 ml	
	High	Low
	buffer medium	buffer medium
	mg	mg
NaHCO ₃	187.5	52.5
Na ₂ HPO ₄		4.4
NaH ₂ PO ₄ · H ₂ O	11.1	
KH ₂ PO ₄		5.2

was 7.6-7.8. The cells were implanted in the low buffer medium and this medium was exchanged once, 3-4 days after preparation of the tissue culture. As the cell sheet developed, the pH progressively dropped, so that after 5-7 days, when the monolayer was complete, the pH was 6.6-6.8. The medium was replaced at time of virus inoculation with a maintenance medium consisting of either the high buffer or low buffer medium. Table I compares buffer systems in the 2 media, as used in this study. The essential difference between the 2 media is the greater content of NaHCO₃ in the highly buffered medium. Tissue cultures in the high buffer medium maintained a pH of 7.6-7.8 during the 7-9 day observation period. However, when the low buffer medium was employed as maintenance medium, the pH of the culture fluid rapidly declined to 6.6-6.8 after 48 hours incubation, remaining at 6.6 during the remainder of observation period. Cells were readily maintained in good condition in both media. In the low buffer medium, the cells tended to appear spindle-shaped, granular and somewhat contracted, whereas cells maintained in the high buffer medium were flatter, more translucent and squamous in appearance. Virus titration endpoints were calculated according to the method of Reed and Muench(9), and were expressed as log TCD₅₀/0.1 ml. Usually 5 tissue culture tubes were inoculated per dilution, but in a few experiments, 2 or 3

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† Markle Scholar in Medical Science.

TABLE II. Comparative Virus Titrations in Low Buffer and High Buffer Media.

Virus	Medium (buffer)	Virus titer							
		Days							
		1	2	3	4	5	6	7	9
<i>ECHO 6</i>									
D'Amori*	L†	3.5‡	5.5	6.5	6.6	6.8	7.2	7.2	7.2
	H	4.5	6.5	6.8	7.0	7.0	7.0	7.0	7.0
Patient No. 8 (Buffalo)	L	.5	1.2	2.5	2.5	2.8	4.0	5.2	5.4
	H	1.2	2.5	4.5	4.6	5.5	5.6	5.6	
14	L	.5	.8	2.6	2.6	3.4	4.0	5.5	5.5
	H	1.4	1.8	4.2	4.8	5.6	5.6	5.8	
18	L	2.4	3.6	4.5	4.6	5.5	5.5	6.2	6.5
	H	3.2	4.5	6.4	6.6	6.8	7.2	7.2	
25	L	.0	.0	.5	1.5	1.5	3.0	3.0	
	H	.0	.5	1.5	2.5	4.0	5.0	5.0	
<i>ECHO 12</i>									
Travis 2-85*	L	2.6	4.8	5.5	5.5	5.5	5.5	5.5	5.5
	H	3.2	4.8	5.5	5.5	5.5	5.5	5.5	5.5
<i>Unidentified orphans</i>									
Patient No. 32	L	1.5	2.5	2.5	3.5	4.2	4.6	5.5	6.4
	H	.8	2.5	4.2	4.5	5.5	5.5	5.8	6.2
801	L		<1.5	<1.5	<1.5	<1.5	<1.5	2.8	
	H		1.8	3.3	4.8	4.8	5.3	5.3	
<i>Poliovirus</i>									
Type I (Mahoney)	L	3.6	6.5	6.8	6.8	6.8		6.8	
	H	3.5	6.3	6.6	6.6	6.6		6.6	
Type II (MEF-1)	L	3.5	6.2	6.5	6.6	6.8		6.8	
	H	3.5	5.8	7.3	7.3	7.3		7.3	

* Prototype strain.

† L = Low; H = High.

‡ Log TCD₅₀/0.1 ml.

tubes were used. A virus† inoculum of 0.1 ml was added to 0.9 ml of the maintenance medium.

Results. Virus titration endpoints were compared in tissue cultures maintained in low and high buffer media. Depression of virus cytopathogenic effect demonstrated in the low buffer medium as compared with the high buffer medium will be referred to as the "pH effect." The results, using prototype and Buffalo isolates of ECHO 6, prototype ECHO 12, unidentified agents Nos. 32 and 801 and poliovirus types I and II, are pre-

‡ ECHO virus prototypes were obtained through the courtesy of the members of the Committee on ECHO viruses(2). In addition, orphan virus isolates from patients with aseptic meningitis were used, including 4 strains of ECHO 6 and 2 unidentified agents, Nos. 32 and 801. These agents were isolated in Buffalo, N. Y., in 1955(5). Coxsackie B1 (Conn-5) was obtained from Dr. A. J. Pappenheimer, Boston, and poliovirus types I (Mahoney) and II (MEF-1) were obtained from Connaught Medical Research Laboratories, Toronto.

sented in Table II. ECHO 6 prototype, as well as the four 1955 Buffalo ECHO 6 isolates, showed a slower rise in titer in the low buffer medium as compared with high buffer medium, with the final titer tending to be lower in low buffer medium. All isolates of ECHO 6 were not equally sensitive to the "pH effect." The unidentified orphan isolate from patient No. 801 showed the greatest "pH effect" of those tested. Poliovirus type I maintained approximately the same titer in both media, whereas type II had a slightly lower titer (0.5 log TCD₅₀) in low buffer medium. Depression of virus titer in low buffer medium was more marked with the more slowly cytopathogenic agents than with viruses which attained high titer rapidly. The "pH effect" was particularly manifest on or about the 5th day of incubation. When incubation was continued sufficiently long, the final titers were frequently similar in both media.

To further clarify the influence of acid pH on virus titer, the low buffer medium was ar-

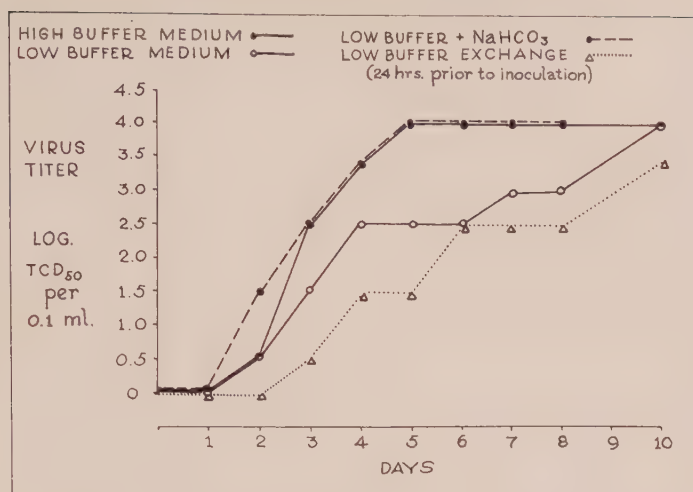


FIG. 1. Influence of alteration of acidity of tissue culture medium upon "pH effect".
Virus: ECHO 6 (No. 25).

tificially maintained at pH of 7.6 by addition of NaHCO_3 to culture tubes during incubation. After incubation for 12 hours, NaHCO_3 (0.5 mg) was added to each tube. The pH was returned from 7.0 to 7.6. After incubation for an additional 36 hours, the pH had dropped to 7.0 and NaHCO_3 (0.5 mg) was again added. The pH was thus restored to 7.6, where it remained for duration of experiment. Titration results in the above cultures were compared to those obtained with cells maintained in low buffer medium and high buffer medium. There was no difference in titration endpoints between high buffer and low buffer medium with added NaHCO_3 , which indicated that the return to an alkaline pH by simple addition of NaHCO_3 prevented the "pH effect." Exchange of tissue culture fluid with low buffer medium 24 hours prior to virus inoculation rather than simultaneously with virus inoculation tended to exaggerate the "pH effect." In this case, the pH of medium at time of virus inoculation was 6.8, as a result of 24 hours incubation. Thus, less than 24 hours after virus inoculation, the pH had reached 6.6. Results obtained employing the above methods of altering "pH effect" are plotted in Fig. 1.

It was found that when glucose, which is present in concentration of 100 mg% in both media, was omitted from low buffer medium,

the pH did not decline. This suggested that formation of acidity was related to glucose metabolism. Cells maintained in low buffer medium without glucose remained in a satisfactory condition for virus study for 7-9-day period. During the period of incubation, the cells without glucose morphologically resembled those maintained in high buffer medium. ECHO 4 prototype was titrated in low buffer medium and in the same medium devoid of glucose. The results are plotted in Fig. 2. The titers obtained with low buffer medium without glucose were comparable to titers obtained with high buffer medium, which served as a control, and in both systems, the pH did not fall below 7.4. In low buffer medium containing glucose, where pH became 6.6 on incubation, the "pH effect" was quite marked. A similar experiment, performed with pH sensitive No. 801 isolate, showed the same effect. The above results again pointed to the fact that it was the development of acidity in tissue culture medium which was associated with depression of virus cytopathogenicity.

The influence of size of virus inoculum on "pH effect" was examined by studying the effect of varying virus doses upon extent of cell damage, rather than by comparative virus endpoint titrations. Degrees of cell involvement as a result of progressive viral infection

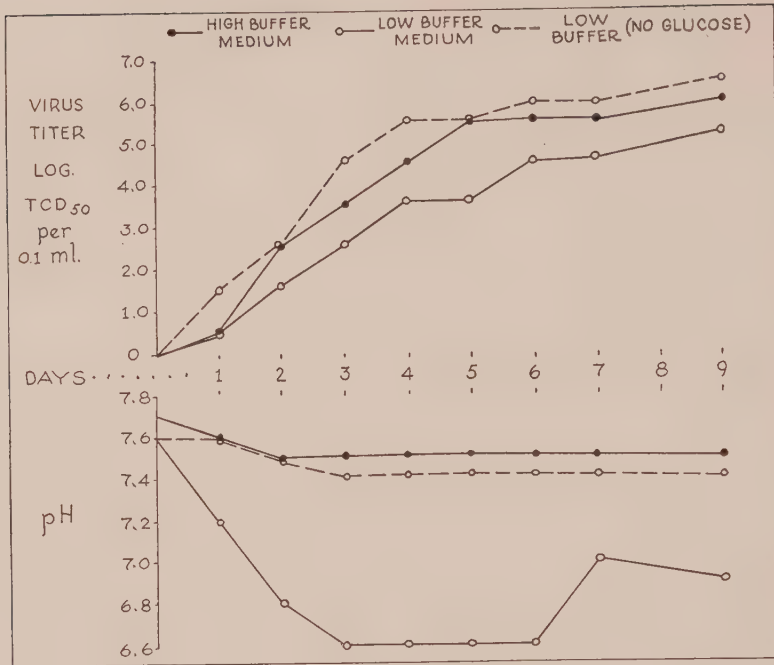


FIG. 2. Effect of omission of glucose from low buffer medium on pH and on growth of ECHO 4 virus.

were arbitrarily rated in 0.5 unit increments from 0.5 (minimal early effect) to a maximum of 3.5 (virtual disappearance of cells). A cytopathogenic score per tube was calculated by dividing the sum of ratings of tubes by the number of tubes in experimental group

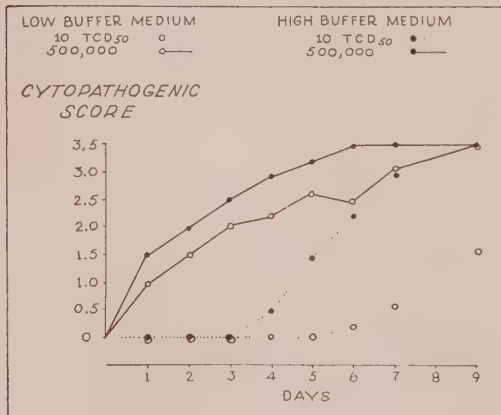
$$\left(\text{Cytopathogenic Score} = \frac{\text{Sum of Ratings}}{\text{No. of tubes}} \right).$$


FIG. 3. Influence of virus dose on "pH effect". Virus: ECHO 6 (No. 8). Cytopathogenic score was calculated according to the formula in the text.

When a large dose of ECHO 6 isolate No. 8 was employed, approximately 500,000 TCD₅₀, the "pH effect" was minimal. When a small dose of the same virus was employed, approximately 10 TCD₅₀, the effect was more pronounced (Fig. 3). With the small virus dose, using low buffer medium, there was no evidence of viral cytopathogenic effect until the 6th day. Cell destruction in this medium, as estimated by the cytopathogenic score, was not very marked even after 9 days incubation. In high buffer medium, the cytopathogenic effect was detected on day 4 and was quite marked on day 7.

Employing the cytopathogenic score technique, the effect of an acid environment was studied with 12 of the ECHO prototypes by inoculation of approximately 100 TCD₅₀ into tissue cultures maintained in low and high buffer media. The results are summarized in Table III. ECHO 1, 5, 7, 8 and 12 showed minimal "pH effect." ECHO 2, 6, 11 and 13 were moderately affected, whereas 3, 4 and 9 were markedly sensitive. Coxsackie B1 was markedly sensitive, while poliovirus types I and II showed little "pH effect."

TABLE III. Sensitivity of Viruses to "pH Effect" as Measured by Cytopathogenic Score.

Virus*	Medium (buffer)	1	2	3	4	Days 5	6	7	8	9
<i>Minimal</i>										
ECHO 1	L†	.2‡	1.5	2.5	3.0	3.5				
	H	.8	1.5	2.9	3.5	3.5				
5	L	.0	.5	1.9	2.5	3.4	3.5			
	H	.2	1.0	2.1	3.2	3.5	3.5			
7	L	.9	2.0	3.0	3.3	3.3	3.5			
	H	.5	2.5	3.5	3.5	3.5	3.5			
8	L	.5	.8	1.6	3.0	3.4	3.5			
	H	.2	1.2	2.5	3.5	3.5	3.5			
12	L	.2	1.1	2.4	3.0	3.5				
	H	.4	1.4	2.5	3.4	3.5				
<i>Moderate</i>										
2	L	.1	.3	1.0	1.5	2.5	3.0	3.5		
	H	.1	1.0	1.7	2.3	3.5	3.5	3.5		
6	L	.0	.3	1.5	2.2	3.0	3.5			
	H	.0	1.0	1.9	2.8	3.5	3.5			
11	L	.1	.5	.6	1.5	2.5	3.1	3.5		
	H	.0	.9	1.4	2.8	3.5	3.5	3.5		
13	L	.3	1.9	2.5	3.0	3.3	3.5			
	H	1.0	2.0	3.0	3.5	3.5	3.5			
<i>Marked</i>										
3	L	.2	.3	1.0	1.5	2.5	3.0	3.5		
	H	.7	1.5	1.9	2.8	3.5	3.5	3.5		
4	L	.0	.1	.2	.8	.8	1.4	2.3	2.6	3.5
	H	.0	.8	1.9	2.8	3.5	3.5	3.5	3.5	3.5
9	L	.0	.1	.4	1.4	2.3	2.5	3.4		
	H	.0	1.0	1.8	2.4	3.0	3.4	3.5		
Coxsackie B1 (Conn-5)	L	.1	.5	.4	.4	2.0	1.6	2.3	2.5	2.7
	H	.0	.6	1.2	1.8	2.5	2.9	3.5	3.5	3.5
Poliovirus Type I	L	.1	1.5	3.0	3.5	3.5		3.5		
	H	.0	1.5	3.4	3.5	3.5		3.5		
" " II	L	.0	1.0	2.5	3.0	3.5		3.5		
	H	.0	1.0	3.5	3.5	3.5		3.5		

* Virus dose, approximately 100 TCD₅₀ score (refer text).

† L = Low; H = High.

‡ Cytopathogenic

The effect of pH of medium upon direct isolation of cytopathogenic enteric viruses from clinical specimens was considered. Known virus-positive stool suspensions were inoculated in parallel into tissue cultures maintained in low buffer and high buffer media. Preparation of stool suspensions and method of inoculation have been described elsewhere(5). The results of this isolation study are presented in Table IV. After a single passage, in high buffer medium, 77% (151/196) of tissue cultures were recorded as positive (*i.e.* showing definitive cytopathogenic effect typical of viral infection), whereas only 39% (87/222) of cultures were positive in low buffer medium. Tissue cultures

with any suspicion of possible virus effect were recorded as questionable. Using these criteria of grading, it was felt that many questionable cultures would be regarded as negative in routine diagnosis, particularly if only a single tissue culture passage were employed.

Discussion. Our results have indicated that development of an acid environment in tissue culture fluid may delay or depress the appearance of viral cytopathogenic effect with some human enteric agents. When the acid environment was neutralized by addition of NaHCO₃ or prevented from forming by omission of glucose, the "pH effect" was not observed. Recently Gifford *et al.*(10) have reported studies on the effect of pH on metabo-

TABLE IV. Influence of "pH Effect" on Recovery of Orphan Viruses from Stool Suspensions.

Virus isolated	No. stools	Low buffer medium			High buffer medium		
		No. of tubes			No. of tubes		
		Pos.	Quest.	Neg.	Pos.	Quest.	Neg.
ECHO 6	19	60	84	42	123	20	25
Unidentified Orphan	3	27	9	0	28	0	0
Total %	22	(222) 87 39	93 42	42 19	(196) 151 77	20 10	25 13

lism of HeLa cells, which showed that increase in acidity decreased poliovirus production. A small virus dose of 25 TCD₅₀ was employed in their study. These investigators found that there was no decrease in virus stability at lower pH levels to account for the decrease in virus production. This would suggest that the "pH effect" is not restricted to monkey kidney cell cultures alone. Although the effect of H⁺ ion concentration on viral infection of cells in culture has not been the subject of intensive investigation, Hanks (11), in an excellent account of nutrition of cells in culture, referred frequently to the fact that of all metabolic endproducts resulting from cellular metabolism, only the H⁺ ion is known to be toxic. The mechanism of H⁺ ion toxicity for cells has not been fully explained.

In our experiments, several factors influencing the "pH effect" became apparent. There were differences in "pH effect," both between ECHO virus serotypes, as well as among different isolates within a single serotype. Rapidly cytopathogenic agents, as typified by the poliovirus group, demonstrated minimal "pH effect." The employment of a large virus inoculum was found to obscure the "pH effect." An explanation for the minimal "pH effect" of rapidly cytopathogenic agents or of large inocula is not entirely clear. Certainly one factor may be the 24-48 hours lag period for production of an acid environment. During this interval a rapidly multiplying virus or a massive virus inoculum may have sufficient time in an environment with a physiological pH in which to cause cell destruction. The enhancement of the "pH effect" by media exchange 24 hours prior to virus inoculation would point in this direc-

tion. Preliminary experiments would suggest that the inhibitory effect of lowered pH is on the host cell, rather than on the free virus, and that virus release into the medium is correlated with the degree of cell damage.

Primary isolation attempts, in a single tissue culture passage, using known positive stool suspensions, revealed that acid producing low buffer medium was approximately one half as effective in detecting definitive viral cytopathogenic effect as the high buffer medium. Consequently, clinical sources of virus material, such as throat washings, spinal fluid or stools containing a small number of infectious units of a pH sensitive agent may falsely be considered virus negative if sought for in the presence of a highly acid medium. Since the isolation of orphan viruses rests entirely upon manifestation of a cytopathogenic effect in tissue culture, the influence of the "pH effect" upon sensitivity of this system appears to be worthy of consideration.

Summary. 1. The cytopathogenic titer of certain orphan viruses in monkey kidney tissue culture was depressed or delayed when grown in the presence of a medium which developed an acid pH. This phenomenon has been termed the "pH effect." 2. Sensitivity to the "pH effect" appeared to be strain characteristic. Differences occurred among serotypes as well as within serotypes of the orphan viruses. One Coxsackie group B virus tested also demonstrated the "pH effect." Two poliovirus types tested were minimally affected by pH variation. 3. Neutralization of tissue culture acidity with an excess of NaHCO₃, or prevention of acid formation by omission of glucose restored optimal viral cytopathogenicity. 4. The "pH effect" tended to be less evident with rapidly cytopathogenic

agents or in the presence of large inocula. 5. Rate of primary isolation of orphan virus from stool suspensions was substantially decreased when acid producing tissue culture medium was used.

The authors wish to acknowledge the technical assistance of Miss Alicia Bukowski.

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Assay of Properdin in Biological Fluids.* (22959)

EARL G. McNALL. (Introduced by V. D. Newcomer)

Department of Medicine, University of California Medical Center, Los Angeles.

A naturally occurring serum protein, properdin, has been described which participates in certain types of non-specific immunity (1). The known constituents of the properdin system are magnesium ions, complement (C'), and properdin (P) (1,2). In the presence of magnesium ions, properdin complexes with zymosan, a complex of insoluble polysaccharides derived from yeast cell wall, and various other polysaccharides (1-4). The third component of complement (C'3) is specifically inactivated by this properdin-zymosan complex. Various assays for properdin based on inactivation of C'3 have been reported (1-3,5). These employ two reagents, (a) a serum depleted in properdin but containing all components of complement (RP) and (b) a serum lacking properdin and C'3 but containing a large excess of all other components of complement (R3).

Since the source of human sera for preparation of satisfactory test reagents is re-

stricted to 10 to 20% of the population, surveys of large numbers of sera are necessary to obtain reagent sera in practical quantities (1,2). The previously described method for removal of properdin from RP preparation has not been demonstrated to be quantitative in our laboratory and complete removal of C'3 from human sera has also been difficult to achieve. In previously described properdin assay there is no provision for the contribution made by C'3 of the test serum, and variable end points result when large particles of zymosan sediment to bottom of the tube. This paper reports a simple reproducible method for preparation of 2 reagent sera and a more sensitive assay for properdin in biological fluids.

Materials. A. *Barbital Buffer*. The composition of stock pH 7.4 buffer was reported elsewhere (2). The diluted buffer was prepared from stock daily and discarded after 12 hours to ensure constancy of pH. B. *Activated Sheep Cells*. Sheep blood in equal volume of Alsever's solution was obtained

* This investigation was supported by Office of Surgeon General, Department of Army.

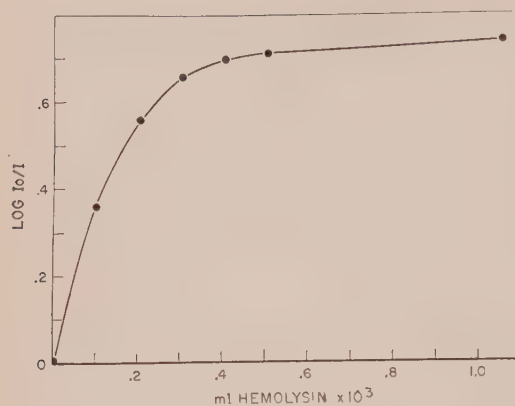


FIG. 1. Hemolysin titration curve.

from commercial supply house. The sheep cells were washed 3 times with 10-15 volumes of barbital buffer. A 4% suspension of washed cells was made in barbital buffer. Hemoglobin concentration of red cells was determined after hemolysis in Spectronic "20" spectrophotometer at $540\text{ m}\mu$. The red cell suspension was then diluted until concentration of cells was exactly 4% by reference to a curve relating number of red cells to hemoglobin. Activated sheep cells were prepared by adding one volume of barbital buffer containing a zero order excess of hemolysin as determined in titration of hemolysin below.

C. Titration of Hemolysin. Varying amounts of 1:1000 hemolysin were added to a series of photometrically standardized 13×100 mm. test tubes containing 0.5 ml of sheep cells. The tubes were incubated at 37°C for one-half hour. 1 ml of a 1:50 dilution of guinea pig complement or RP + R3 (1:50 each) prepared in 0.5% bovine serum albumin in barbital buffer was then added. The tubes were again incubated for one-half hour at 37°C ; 0.5 ml of versene was added to stop the reaction. The tubes were centrifuged at 1,000 to 1,500 $\times g$ for 10 minutes and read at $540\text{ m}\mu$ (Fig. 1). Concentrations of hemolysin beyond 1:2000 do not cause appreciable changes in extent of hemolysis. Each new lot of hemolysin must be standardized.

D. Versene. 0.1% Versene (N, N' ethylene diamine tetra-acetic acid) was made in physiological saline and adjusted to pH 7.4.

E. Zymosan. Commercially obtained zymosan[†] was ground to a

fine, easily dispersible powder with mortar and pestle and suspended uniformly in barbital buffer. Concentration of zymosan routinely used in the test was established by determining the zero order range under conditions of the test (Fig. 2). Each new lot of zymosan must be standardized.

F. Inulin. Commercially obtained inulin[‡] was ground as in E. Vigorous shaking was required to disperse small clumps in some preparations. Concentration as in E (Fig. 2). Each new lot of inulin must be standardized.

G. Serum. Syringes, needles, and all glassware, see Methods section. Blood was allowed to clot at room temperature for one or two hours or overnight in refrigerator. Clear, unhemolyzed sera, animal and human, were stored at -20°C . No loss of properdin activity took place for at least two months. Serum was

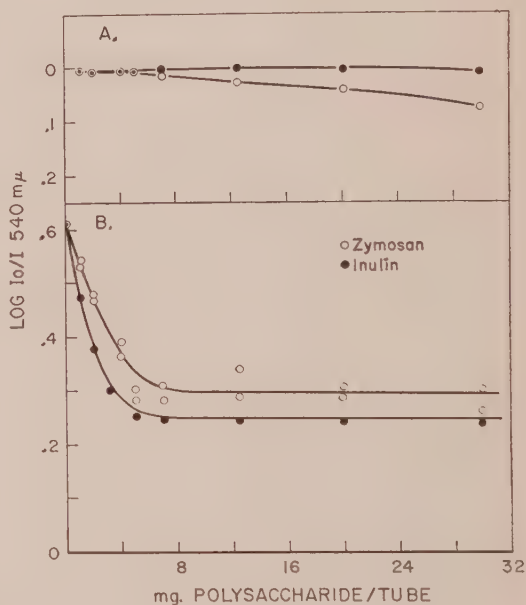


FIG. 2. A. Effect of inulin and zymosan on guinea pig RP in absence of properdin, comparing extent of C' binding by the 2 polysaccharides. B. Effect of inulin and zymosan on guinea pig RP in presence of properdin contrasting reliability of the calibration curve readings.

[†] Zymosan Lot #B-61 was obtained from Immunological Specialties, Los Angeles, California. Zymosan Lot #5B171 was obtained from Fleishman Laboratories, Standard Brands, Stamford, Conn.

[‡] Inulin was obtained from Matheson Co., East Rutherford, N. J.

thawed and diluted with chilled barbital buffer immediately before assay. Human serum was diluted 1:50; rat was diluted 1:200; mouse was diluted 1:50. H. *Properdin* was obtained from normal human serum by methods reported elsewhere(2). I. *RP* is prepared from commercial guinea pig complement by slowly adding and stirring 4 mg of finely powdered zymosan for each ml of serum. The serum-zymosan suspension is stirred continuously during one hour incubation at 20° to 25°C. The resulting suspension is then centrifuged at 1000-1500 X g for 10 minutes. The precipitate is discarded and the process repeated twice. In the last incubation the temperature is maintained at 37°C for 20 minutes and centrifuged for 15 minutes at 1000-1500 X g. The supernatant is frozen in vials in one or two ml volumes at -20°C. No loss in activity took place with samples continuously frozen for 2 months. J. *Properdin-zymosan complex*. Human serum containing 4 mg of zymosan/ml was incubated with constant stirring for one hour at 20°C and centrifuged at 1000-1500 X g for 10 minutes. The supernatant was discarded. The precipitate was washed 2 times with pH 6 phosphate buffer as reported elsewhere(2). K. *R3* is prepared under the same conditions as *RP*, except that temperature of incubation is 37°C throughout and during the first incubation instead of zymosan the properdin-zymosan complex is used in excess to ensure complete removal of C'3. Although guinea pig sera are routinely used for *R3* preparations, we found that human *R3* can be made by this process. All our attempts to deplete human sera of C'3 have been successful. The C'2 component titers of some sera are not, however, sufficiently high to be useful for *R3* preparation.

Methods. Since chromic acid washed glassware occasionally contains sufficient chromate ions to inactivate complement, all glassware was washed in trisodium phosphate solution and rinsed with triple distilled water. All incubation at 37°C took place in constant temperature circulating water bath. Each reagent used was individually standardized. In addition to determining the C'3 titer of *RP*

TABLE I. A Typical Properdin Assay Protocol.

Tube	Serum 1:50	Bu- fered saline	RP + R3 (1:50 each)	Inulin	Code
1	1.0	.5	1.0		SRPR3
2	1.0		1.0	.5	SRPR3I
3	1.0	1.5			S
4		1.0	1.0	.5	RPR3I
5		2.5			
6		2.0		.5	I
7		1.5	1.0		RPR3
8		2.5			
9		2.3	.2		
10		2.1	.4		
11		1.9	.6		
12		1.7	.8		
13		1.5	1.0		
14		1.3	1.2		
15		1.1	1.4		

and *R3*, the C'1, C'2, C'4 titers were also determined for each lot. It has been our experience that activity of *RP* and *R3* does not change measurably for at least 2 months continuous storage at -20°C.

Properdin assay procedure. 1. Serum to be tested is diluted with chilled barbital buffer immediately prior to test. 2. 1 ml of the diluted test serum is added to tubes as indicated in protocol (Table I). 3. 0.5 ml of inulin or zymosan is added to tubes which are shaken briefly to suspend inulin or zymosan uniformly. 4. Saline is then added to make the volume 1.5 ml in each tube. 5. 1 ml of *RP* + *R3* is added to test and varying amounts of *RP* + *R3* is added for control tube 8 through 15. 6. Incubate at 37°C in circulating water bath for 1½ hours. 7. Centrifuge at 1000-1500 X g for 10 minutes to remove properdin-inulin- (or zymosan) C'3 complex from supernatant. 8. 2 ml of activated sheep cells are added with gentle shaking so that properdin-inulin-C'3 button is not disturbed and tubes incubated at 37°C for one-half hour. 9. 0.5 ml of 0.1% versene solution is added to each tube with gentle mixing. 10. Centrifuge immediately at 1000-1500 X g for 10 minutes. 11. The hemoglobin liberated in the supernatant is read at 540 mμ.

The difference between *SRPR3* and *SRPR3I* represents the decrease in C'3 concentration. This decrease is equivalent to the properdin concentration and therefore

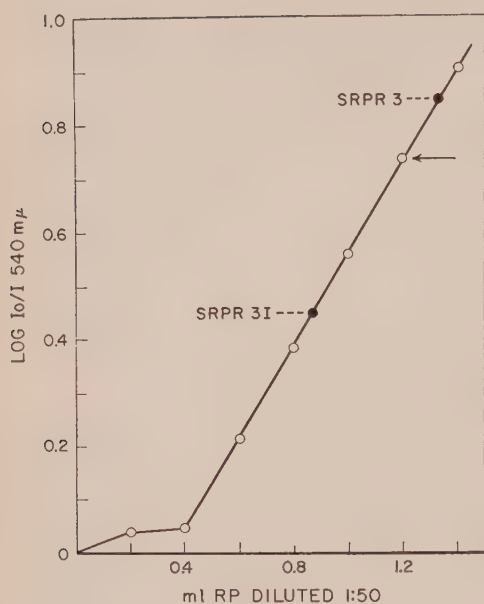


FIG. 3. Relation between extent of hemolysis and varying amounts of RP + R3 and readings of a typical test sera. Arrow indicates level of RP + R3 which corresponds to 50% hemolysis.

properdin concentration may be expressed as a function of optical density difference (Fig. 3).

The optical density of SRPR3 may be taken as 100% C'3. The decrease of C'3 activity (SRPR3I reading) is expressed in percent of SRPR3 optical density reading.

The plot of RP + R3 against optical density (Fig. 3) is a control to ensure that all components except C'3 are in zero order excess. When this is true, a linear plot is obtained between 10% hemolysis and 80% hemolysis. The RP + R3 concentrations selected for the test fall into the range between 10 and 60% hemolysis. If components other than C'3 are limiting, a curve rather than a linear dependence will result.

If C'3 is limiting in RP + R3, a straight line passing through the origin is obtained in a plot of SRPR3-SRPR3I versus ml of serum (or properdin) (Fig. 4). If residual C'3 is still present, the line will not pass through the origin but will intercept the SRPR3-SRPR3I at a point above the origin. If a component other than C'3 is limiting in RP + R3, this plot will pass through a point

below the origin. When both these conditions occur together, a curve results.

The properdin content of 1 ml of 1:50 human serum diluted with barbital buffer generally falls into upper region of test range. Sera containing higher or lower properdin levels can be appropriately diluted to fit into the test range. If the dilution is carried out immediately following thawing and just prior to the test, no discernible effect on dilution is obtained. Data obtained from direct pipetting micro-quantities, *i.e.*, 0.02 ml, give the same properdin level as 1 ml of 1:50 dilution of the same serum.

Since in the test SRPR3 contains both complement content of RP + R3 and the complement present in test serum, it was necessary to determine whether the complement content of human test serum and the guinea pig RP + R3 were equivalent in such mixtures. Secondly, it is important to establish what effect, if any, is observed in the C'3 inactivation of mixtures of sera obtained from the two sources. In all cases there was an

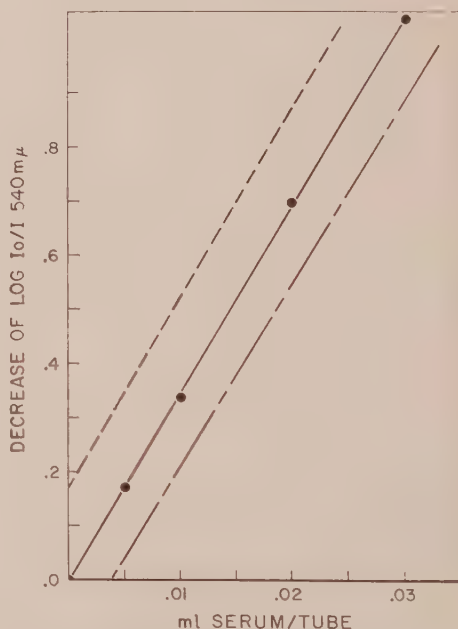


FIG. 4. Relationship between the SRPR3-SRPR3I of a serum containing properdin and the ml test serum. Dash line indicates RP + R3 not completely depleted of properdin; interrupted line indicates RP + R3 containing an excess C'3 (not in limiting concentrations).

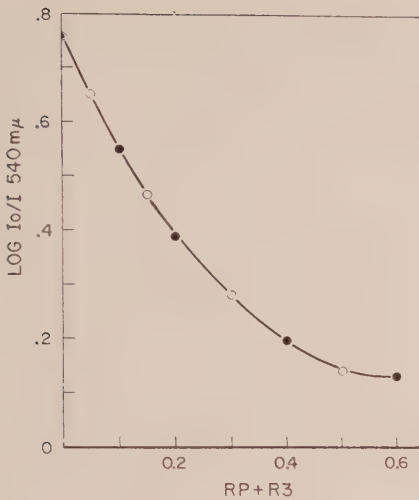


FIG. 5. Rates of inactivation of C'3 by human properdin in ● human RP + R3 and ○ guinea pig RP + R3. Human RP + R3 was diluted (1 : 20) to give the same density as guinea pig RP + R3 (diluted 1 : 50).

additive effect between the 2 complements in the SRPR3. To establish whether there was any difference in C'3 inactivation from RP + R3 preparations obtained from the 2 sources, varying concentrations of mixtures of the 2 preparations were used. This experiment is summarized in Fig. 5.

Discussion. Guinea pig serum was routinely used as the source for RP and R3 reagents utilized in the test. The advantages of using this serum are a) guinea pig serum has the lowest properdin content of any experimental animal(1), and b) the limiting component in guinea pig complement is C'3 (6). With the exception of C'2 the other components are in large excess so that most RP preparations do not actually require addition of R3 reagent. To ensure that C'2 does not become limiting, an equal volume of R3 is always added as supplement to the RP reagent.

Human serum has a much higher properdin level than that of guinea pig, and C'3 is usually not the limiting component(1,6). It is, therefore, difficult to obtain human sera to make satisfactory RP and R3 reagents as described in the literature. To obtain a suitable human RP, it is necessary to use sera which originally contain higher than average

levels of C'2 and lower than average levels of properdin. We found that satisfactory R3 reagents can be made with human sera from at least 50% of random human sera tested utilizing incubation with an excess of PZ complex.

Tube (S), which is the test serum alone, reflects the overall C' titer of this serum. The difference between SRPR3 and RPR3 reflects the C'3 titer of the test serum. Occasionally the contribution of C'3 made by the test serum is high relative to the properdin content; therefore, without above controls on complement derived from the test serum, error would arise.

Zymosan varies from lot to lot in activity of properdin binding as well as in inactivation of C'3. There is extremely wide variation between duplicate tubes when the coarse zymosan preparation described in the literature is used. Finely powdered zymosan gives more consistent readings.

Inulin binds properdin and inactivates C'3 in a manner similar to zymosan. Since this polysaccharide is chemically descript and can be uniformly suspended in barbital buffer, inulin can be used in higher concentrations than zymosan. It does not absorb out other components of complement as is frequently the case with zymosan in high concentrations (Fig. 2). Inulin is used in this test as the C'3 inactivating polysaccharide in preference to zymosan.

Spectrophotometric determination of hemolysis is a sensitive and highly reproducible means of quantitating properdin and complement activity since an infinite number of concentrations can be extrapolated on the linear plot relating C'3 activity to properdin activity as well as C' activity of the test serum. A single tube establishes a finite concentration of C'3 in the absence of inulin and another tube establishes its concentration in the presence of inulin. Two tubes (run in triplicate) give the properdin activity of a single serum.

The properdin content of serum that shows anticomplementary activity can be established by a series of dilutions of this serum in the presence and absence of known amount of

purified properdin to determine recovery value of the added properdin. This recovery value can then be used to calculate the true properdin content of such serum.

Summary. 1. A sensitive, reproducible assay for properdin is described using spectrophotometric determinations of C'3 content. 2. RP and R3 reagent sera for the properdin assay may be readily prepared from guinea pig serum. 3. RP and R3 from both human and guinea pig sources may be used interchangeably in the reported properdin assay. 4. Inulin was found to be more satisfactory than zymosan as a properdin binding and C'3 inactivating agent.

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Isolation of Anticoagulant Fractions from Crude Fucoidin. (22960)

GEORG F. SPRINGER, HAROLD A. WURZEL, GEORGE M. MCNEAL, JR., NORMA J. ANSELL
AND MARY F. DOUGHTY. (Introduced by Colin M. MacLeod)

William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Phila.

Studies on methylpentose-containing polysaccharide material of plant origin have been in progress in this laboratory for more than two years(1,2). During this work, it occurred to us in view of the known anticoagulant activity of some other sulfated polysaccharides(3), that fucoidin, a polymer of L-fucose monosulfate, present in large amounts in the brown marine algae *Phaeophyceae*, might possess anticoagulant activity. This was found to be the case. The present paper describes the preparation of fucoidin fractions and gives data on some of their chemical properties and anticoagulant activities.

Material and methods. Source and preparation: Air dried *Fucus vesiculosus* was obtained from Woods Hole Marine Laboratories, Mass., between April and July, 1956. The preparation of crude material was based on older procedures(4). Purification procedures were guided by determinations of the anticoagulant activity employing the protamine titration test. Crude fucoidin was prepared as follows. About 1 kg dried *Fucus vesicu-*

losus was ground for 28 hrs. in a ball mill. To the powder obtained, 4 l of tap water were added and the mixture heated with occasional shaking in boiling water bath for 18 hrs. Toluene was added and the material left at room temperature overnight. The supernatant fluid amounted to about 500 ml to which 1 l of water was added. The fluid was then freed of insoluble matter by filtering through muslin. One hundred grams of lead acetate in 300 ml water were added and the precipitate removed by filtration. Barium hydroxide was added to the supernate until it became pink to phenolphthalein. The resulting precipitate was allowed to settle, the supernate siphoned off, discarded, and 300 ml of 4N H₂SO₄ were added for every 700 ml of precipitate containing fluid. The mixture was then shaken mechanically 4 hrs. This was followed by dialysis for 3 days against running tap water. The nondialyzable material was concentrated *in vacuo* to 500 ml, freed of insoluble material by spinning at 12000 g for 45 min. and the supernate poured into 10 vol. of 100% ethanol containing 0.3% sodium acetate. The precipitate was washed with

* This work has been aided by grant from Ciba Pharmaceutical, Inc., Summit, N. J.

TABLE I. Fractionation of Crude Fucoidin at Various Concentrations of Ethanol.

Fraction	% ethanol	% methylpentose	% recovery
I	42	25.8	30
II	47.5	32.2	4.7
III	52.5	44	14.6
IV	59	48.5	14.1
V	63	59	7.3
VI	69	65.5	7.1
VII	78	65.5	9.4

100% ethanol and dried *in vacuo* over P_2O_5 . This material, about 1.5-2.0% of the air dried algae, was designated as crude fucoidin. This crude material was purified by one of the following two methods: a) crude fucoidin was made up to 2% in water and fractionated with ethanol containing 0.3% of sodium acetate. Total recovery based on crude fucoidin was about 87%, which was divided between the different fractions as shown in Table I. b) To 250 ml of a 1.5% solution of crude fucoidin in water, 13 ml of a 10% aqueous solution of cetyl dimethyl benzyl ammonium chloride was added drop wise with continuous stirring. The resulting precipitate was washed with 100% ethanol and dried *in vacuo* over P_2O_5 ; the yield was 2.7 g. A 2% solution was made by dissolving a fine powder of this preparation in 25 ml of 3.6 M aqueous solution of $CaCl_2$; insoluble matter was discarded. The material was fractionated by addition of 100% ethanol. Precipitates obtained at final ethanol concentrations of 45% and 67% were dialyzed and dried as described above. The yield was 32% and 40% respectively on weight basis of crude fucoidin.

Biological determinations: Clotting time, prothrombin consumption, platelet counts, recalcification time, one stage prothrombin, labile factor and protamine titrations were determined according to standard procedures (5). Antithrombin activity of fucoidin and heparin was determined by adding various amounts of these materials to 0.1 ml of a 1% fibrinogen solution (Armour Laboratories). The fibrinogen was then transformed to fibrin by adding 10 units (0.1 ml) of topical thrombin (Parke Davis Laboratories). All biological activities are expressed on the

basis of weight of vacuum-dried (over P_2O_5) material and are compared to those of 1% preparation of Na-heparinate (Abbott Laboratories) which contained 1000 U.S.P. units/ml. Blood employed for *in vitro* studies was obtained from healthy human donors selected at random. Healthy rabbits of either sex weighing 2-3 kg were employed. They were fed an unrestricted diet of Purina chow and water supplemented with fresh carrots.

Physical and chemical determinations. P was determined according to the method of Zilversmit and Davis(6), Ca according to Clark and Collip(7), Na and K by means of Baird flame photometer. Hexosamine estimations were made according to the method of Elson and Morgan(8). Methylpentose was determined spectrophotometrically(9) employing a Beckman DU spectrophotometer. L-fucose (Mann Biochemicals) recrystallized from methanol was used as standard. Reducing sugar was determined by the method of Folin and Malmros(10). Relative viscosity was measured in Ostwald viscosimeter. Hydrolysis of fucoidin was carried out with 1 N H_2SO_4 in sealed tubes at 100°C, the hydrolysates were neutralized with a stoichiometric amount of $Ba(OH)_2$ and concentrated. Paper chromatographic technics employed were those described previously(2).

Results. Fucoidin which precipitated at final ethanol concentration of between 49-64% uniformly was found to be the most active biologically. *All data given in this report refer to fractions obtained in this range of ethanol concentration.*

Anticoagulant activity. Fractions that proved most potent (40-70% of heparin activity) in the protamine titration test were also found to be the most active in all other tests employed.

The *in vitro* inhibition of coagulation of blood and recalcification of plasma are shown in Fig. 1. Fucoidin possesses 85 to over 100% of the potency of heparin in the recalcification test and it has 10% heparin activity in whole blood coagulation inhibiting test. Fucoidin delays action of thrombin on fibrinogen considerably more than does heparin (Fig. 2). As shown in Fig. 3, it is less potent

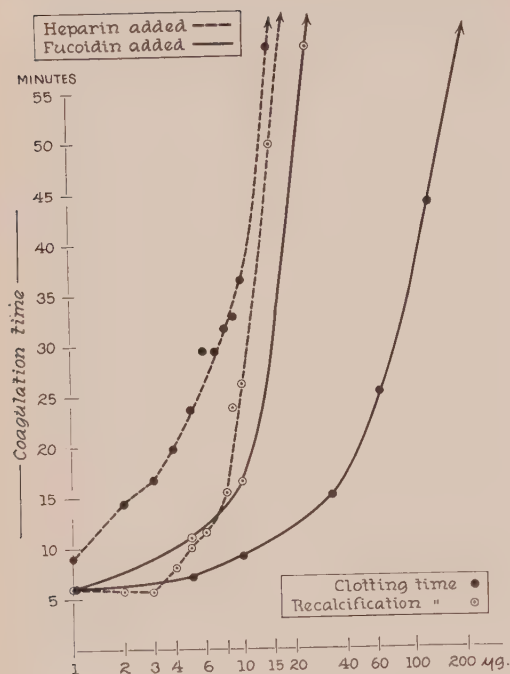


FIG. 1. Influence of fucoidin and heparin on blood coagulation and plasma recalcification.

an inhibitor in the one stage prothrombin test. Fucoidin did not precipitate fibrinogen or fibrin in any of these studies.

The influence of fucoidin on blood clotting mechanism *in vivo* was studied in rabbits. The results obtained in 12 rabbits injected intravenously are listed in Table II. In all rabbits clotting time was prolonged. This effect lasted from $2\frac{1}{4}$ to 10 hours. The average was 6 hours. Even more impressive was the prolongation of recalcification time. From an average of about 4 minutes, it was usually delayed to more than 30 minutes. In many animals, the prolonged recalcification time persisted after clotting time of whole blood had returned toward normal. In five animals, clot retraction was markedly impaired. In one of these, it was still poor after clotting time had returned to normal. A similar observation was also made after heparin administration. One of the 12 rabbits developed transient thrombocytopenia each time that it was injected with the standard dose of fucoidin. After intravenous injection, thrombocytes dropped from pretreatment level of

372,000 to 16,000 at 3 hrs., rose to 66,000 at 6 hrs. and returned to 250,000 at 7 hrs.

Table II further demonstrates a significantly prolonged prothrombin time after injection of fucoidin. At no time was there a demonstrable change in the content of labile factor. Prothrombin consumption was prolonged from an average of 27.5 seconds to 38.2 seconds for 3-4 hours after injection of fucoidin and in general remained prolonged as long as clotting time was delayed.

The anticoagulant effect of fucoidin was undiminished on repeated injections. No rabbit showed toxic symptoms at any time nor was there sloughing at the site of injection, even if the material was given in high doses intramuscularly or extravasated into the soft tissues. There was no impairment of appetite or vitality of the rabbits.

Physical and chemical. All analyses were performed on the most active fraction which had been thoroughly dialyzed against 300 vol. deionized water. Calculations were made after subtraction of 20.45% moisture, which

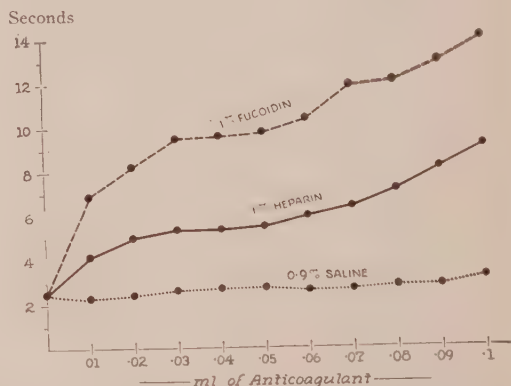


FIG. 2. Inhibition of thrombin-fibrinogen reaction.

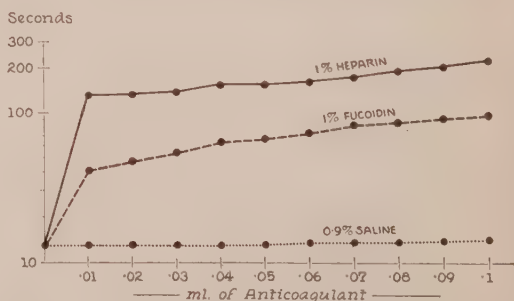


FIG. 3. Action of fucoidin and heparin on one stage prothrombin time.

TABLE II. Influence on Clotting Factors in Rabbits Given 45 to 50 mg Fucoidin Intravenously.

	Normal range	Hr after inj. of fucoidin	
		$\frac{3}{4}$ -1 $\frac{1}{2}$	3-4
Clotting time (min.)	10-25 (19)	39-147 (69)	21-112 (44)
Recalcification time (sec.)	120-390 (205)	(> 2000)	(> 2000)
Labile factor (sec.)	8.3-10.8	7.4-10.9	8.3-10.7
Prothrombin time (sec.) (one stage)	9.2-12 (10.5)	10.5-16.5 (14.1)	10.2-12.5 (12.0)
Platelets	218,000-786,000 (430,000)	136,000-480,000* (310,000)	208,000-464,000* (320,000)
Prothrombin consumption	16.7-42 (27.5)	16.7-63 (33.8)	22-56 (38.2)

Figures in parentheses give arithmetic means.

* One out of 12 rabbits reacted with a thrombocytopenia upon inj. of fucoidin. The effects in this animal are described in text.

could be removed by drying fucoidin over P_2O_5 at 40°C at a pressure of 0.1 mm mercury.

Relative viscosity of a 1% solution of fucoidin in 0.85% saline was $\eta = 1.2$ (35°C). Ultraviolet absorption of 0.8% solution in water showed very weak absorption between 256 and 280 $m\mu$. Otherwise no absorption maximum was found between 220-600 $m\mu$. Specific rotation was $[\alpha]^{25}_D = -119.6^\circ$ (c 1 in water).

The analytical data were: fucose 44.2%, S 9.6%, Na 6.9%, K 0.76%, Ca 1.65%, N 0.71% (micro Kjeldahl), $P < 0.05\%$. Sulfated ash 25%. No hexosamine was demonstrable.

Paper chromatograms of hydrolysates of the most highly purified and potent preparations of fucoidin showed no carbohydrates other than fucose and a component which moved between 2-O-methylfucose and fucose and gave color reactions of methylpentoses (2).

The reducing values of hydrolysates rose rapidly from zero within the first hour of hydrolysis and reached a steady value after about 3 hours.

Discussion. The experiments reported here show that purified fucoidin fractions from *Fucus vesiculosus* are potent inhibitors of blood coagulation *in vitro* and *in vivo*. It is remarkable that the most active material contained after hydrolysis only 2 demonstrable sugars, namely fucose and a faster component located between fucose and 2-O-methylfucose on paper chromatograms (2). In

agreement with Lunde *et al.* (11) but in contradistinction to Percival and Ross (12), the main inorganic ion of fucoidin was sodium.

The methylpentose content of the fractions increased with increasing ethanol concentration (Table I), while the most potent anticoagulant material was obtained at medium alcohol concentrations. Absorption observed in the ultraviolet range (256-280 $m\mu$) indicates a small amount of non-carbohydrate impurity. The sulfur content of active preparations was 80% of that reported for heparin (13).

Fucoidin is characterized by its high moisture content. While the analytical data presented here have been corrected for an easily removable moisture content of 20.45%, Percival and Ross (12) have reported that crude fucoidin, dried as described in this paper, still contains 15.4% moisture.

As shown in this report, fucoidin like other acid polysaccharides (14) combines with certain basic substances. Its anticoagulant activity was neutralized by protamine. Fucoidin is also precipitated and inactivated by the long chain quaternary ammonium salt, cetyl dimethyl benzyl ammonium chloride. Dissociation of fucoidin-base complexes with strong salt led to restitution of activity.

Sulfuric acid esters of carbohydrates other than heparin have previously been employed as anticoagulants. Some of these have been found to be satisfactory substitutes for heparin in anticoagulant therapy such as synthetic dextran sulfate which exhibited no toxic effects (15,16) while others, such as syn-

thetically sulfated polymannuronic acids and polygalacturonic acids have been reported to have certain undesirable side effects(17). Both of these are derivatives of uronic acids.

While it was possible occasionally to obtain a significant effect in rabbits of 2-3 kg weight by giving 15 mg fucoidin intravenously, it was necessary to employ higher concentrations of fucoidin than heparin in these animals to obtain reproducibly significant alterations of the *in vivo* clotting mechanism. When such an effect was obtained it lasted longer than after giving heparin. Fucoidin, however, appeared to be more potent than heparin as an antithrombin.

Some sulfated polysaccharides agglutinate platelets. Grosswall, Ingelmann and Mosimann have shown with sulfated dextrans that this undesirable side effect depends on a certain molecular size(18). Present studies in 12 rabbits showed with one exception, an insignificant drop in platelet count after injection of fucoidin (Table II). An insignificant drop of platelets was also observed in our preliminary studies with heparin in rabbits. The very transitory fucoidin-induced thrombocytopenia in one rabbit may have been caused by platelet agglutination, although we have not demonstrated this as the cause. A similar fall in platelet count has occasionally been observed in patients treated with heparin.[†] The rabbit which developed thrombocytopenia following fucoidin injection did not show a bleeding tendency.

Further chemical and biological studies are required to establish the mode of action and non-toxic nature of fucoidin fractions. It is not known at present whether the "fast moving component" is a part of the active molecule, although this is suggested by paper chromatographic evidence. The low nitrogen content of active preparations is probably due to impurities. Failure to detect hexosamine by the Elson-Morgan reaction suggests the absence of nitrogen-containing sugars and it seems reasonable to assume that the anticoagulant activity is connected with the O-sulfate groups, although all O-sulfated carbohydrates do not possess anticoagulant ac-

tivity (e.g. agar). There is no indication that our purest fucoidin contains uronic acid.

Summary. A method of preparation, some physical and chemical properties and anticoagulant activities of fucoidin fractions from *Fucus vesiculosus* have been described. Certain fractions prepared from crude fucoidin proved to have potent anticoagulant effects. The nature of the anticoagulant action and the chemical composition place fucoidin in the group of heparinoids. The most active preparations contained no demonstrable carbohydrate other than fucose and a component which migrated on paper chromatograms faster than fucose and rhamnose but slower than 2-O-methylfucose. This component gave all color reactions of the methyl-pentoses.

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Factors Influencing Excretory Patterns of Cesium-134, Potassium-42 and Rubidium-86 in Rats.* (22961)

FRANK R. MRAZ AND HOMER PATRICK. (Introduced by S. L. Hansard)

*University of Tennessee - Atomic Energy Commission Agricultural Research Program,
Oak Ridge, Tenn.*

MacLeod and Snell(1) reported that cesium, potassium, and rubidium behaved similarly in nutrition of lactic acid bacteria. These elements are known to enter the solute complex, participating in ion antagonism, osmosis, permeability regulation, maintenance of colloidal state and similar physiological phenomena(2). Mraz *et al.*(3) observed an increase in excretion of cesium-134 by the rat when dietary potassium was increased. Hamilton(4) reported that cesium excretion was distributed equally between feces and urine. Hood and Comar(5) found that the rat excreted 12 times more cesium-134 in urine than in feces. Mraz and Patrick(6) reported that supplementation of a simplified (casein-corn starch) rat diet with natural feedstuffs decreased the ratio of urinary to fecal excretion of cesium-134 and potassium-42.

This study was initiated to ascertain the factors responsible for these changes in excretory patterns of cesium-134 and potassium-42. Rubidium-86 was also used because of its biological and chemical similarity to cesium and potassium.

Methods and materials. The basal diet

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employed was the simplified rat diet used by Mraz and Patrick(6). Vermiculite, alfalfa, beet pulp, bentonite, or Amberlite IRC-50 (cation exchange resin) were incorporated at 10% of the diet substituting for an equivalent amount of corn starch in the basal diet. Two potassium-deficient diets were formulated, one from the basal and the other from the 10% vermiculite diet. Four weanling albino rats (Rockland strain) were used per diet in each experiment. All rats were fed their respective diets for 7 days before subcutaneous administration of 0.5 ml of the radioisotope dosing solutions. This period was considered of sufficient length to permit the rat to adjust to the new diet. Five microcuries (μC) of cesium-134 or 20 μC of rubidium-86 were administered to each rat in the cesium and rubidium studies and a 72-hour balance trial made. The short half-life of potassium-42, 12.5 hours as compared to 2.3 years for cesium-134 and 18.6 days for rubidium-86, necessitated the use of 100 μC of the nuclide and only a 48-hour balance trial. All nuclides were administered in the chloride form. Cesium-134 was used at tracer levels, while potassium-42 and rubidium-86 dosages contained 0.38 mg potassium and 0.063 mg rubidium, respectively. *Feces and urine* samples were collected every 24 hours after administration of the nuclides. Excreta were digested with nitric acid and aliquots counted in scintillation well-type counter. In the interest of ease of recording and reading, fecal, urinary, and total excretory data have

TABLE I. Influence of Some Organic and Inorganic Materials on Excretory Pattern of Cesium-134 in Rats.

Treatment	Excretion*			Ratio of urinary /fecal Cs-134
	Urinary	Fecal	Total	
Basal + K	24.9	1.0	25.9	24.9
" - K	4.4	0.3	4.7	14.2
Vermiculite + K	13.6	25.5	39.1	0.5
" - K	4.3	22.1	26.4	0.2
Alfalfa + K	24.7	2.5	27.2	9.8
Beet pulp + K	21.7	6.2	27.9	3.5
Bentonite + K	14.8	15.4	30.2	1.0
Amberlite IRC-50 + K	22.2	3.2	25.4	7.0
L.S.D.†	8.2	1.4	7.5	

* Expressed as % of subcut. admin. dose/rat.

† Least significant difference between means at 5% level of confidence.

been reported utilizing a maximum of 3 significant figures and are not indicative of sensitivity of the experiments. The ratios were obtained from urinary and fecal excretory data containing 3 significant figures and therefore may differ slightly from the ratios which would be obtained from data reported in the Tables. The 5% level of confidence was the criterion for reporting significance in *in vivo* studies and the 1% level in *in vitro* studies. *In vitro* studies were performed in 125 ml Erlenmeyer flasks. Two flasks were used with 0.5 g of each material (air dried and passed through a sieve, 40 meshes/inch) and 2 were left as blanks to ascertain the influence of adsorption on glass. Twenty-five ml of solutions containing 1 ml of radio-nuclide dosing solution in 499 ml of distilled water or of 0.1 N solutions of either potassium chloride, hydrochloric acid or sodium chloride were placed in each flask. Standards were removed at this time. The flasks were stoppered and shaken 5 times during the first 3 hours and then permitted to stand for an additional 21 hours. Two aliquots of supernatant solution were then removed from each flask and counted against the standards. In all trials it is believed that results obtained from distilled water solutions of nuclides with bentonite would have been lower if some of the bentonite had not been colloiddally suspended.

Results. In the cesium-134 experiment,

Table I, a significant reduction in urinary and total cesium-134 excretion and in the ratio of urinary to fecal cesium-134 was noted in rats fed potassium-deficient basal diet when compared to those fed potassium-adequate basal diet. Potassium deficiency in the 10% vermiculite diet induced significant decreases in urinary, fecal and total excretion of cesium-134, when compared to the potassium-adequate vermiculite diet. The excretory ratio was also reduced. Total excretion of cesium-134 on potassium-deficient diet containing vermiculite was approximately equal to that obtained on potassium-adequate basal diet. Rats fed diets containing alfalfa, Amberlite IRC-50, beet pulp, bentonite, or vermiculite had lower excretory ratios and significantly higher fecal cesium-134 excretion than those fed the basal diet. Both bentonite and vermiculite significantly decreased urinary excretion of cesium-134. The *in vitro* studies with cesium-134, Table II, indicated that vermiculite, alfalfa, beet pulp, and bentonite preferentially removed cesium-134 ions from solutions containing potassium, hydrogen, or sodium ions. Amberlite IRC-50, however, appeared to adsorb ions at random. In the presence of other ions, the order of adsorption by materials was vermiculite > bentonite > beet pulp, which was the same as their order in the *in vivo* studies in Table I.

In the potassium-42 experiment, Table III, a significant reduction in urinary and total potassium-42 excretion, was found in rats fed potassium-deficient diets when compared to those fed potassium-adequate diets. No significant difference in urinary or fecal excre-

TABLE II. Cesium-134 Remaining in Solution Expressed as % of Standard Solutions.

Treatment	Cesium-134 diluent			
	.1 N KCl	.1 N HCl	.1 N NaCl	H ₂ O
None	100	99	96	34
Vermiculite	36	16	6	3
Alfalfa	94	91	82	88
Beet pulp	85	67	49	40
Bentonite	31	31	22	44*
Amberlite IRC-50	98	98	95	10

Least significant difference between means at 1% level of confidence is 2%.

* Supernatant contained suspended particles.

TABLE III. Influence of Some Organic and Inorganic Materials on Excretory Pattern of Potassium-42 in Rats.

Treatment	Excretion*			Ratio of urinary /fecal K-42
	Urinary	Fecal	Total	
Basal + K	15.5	.5	16.1	29.1
" - K	.6	.3	.9	1.9
Vermiculite + K	13.9	1.5	15.4	9.4
" - K	1.1	.6	1.7	1.8
Alfalfa + K	23.6	1.2	24.8	20.5
Beet pulp + K	12.2	2.3	14.5	5.4
Bentonite + K	8.4	3.4	11.8	2.4
Amberlite IRC-50 + K	10.3	5.1	15.3	2.0
L.S.D.†	4.6	1.8	4.3	

* Expressed as % of admin. dose/rat.

† Least significant difference between means at 5% level of confidence.

tion of potassium-42 was observed between rats fed the vermiculite diets and those fed basal diets with comparable potassium levels. The excretory ratios between these two diets containing adequate potassium differed by a factor of three, with vermiculite having the lower ratio. Alfalfa significantly increased urinary and total potassium-42 excretion, but had no significant effect on fecal potassium or on excretory ratio when compared to basal diet. Beet pulp, bentonite, and Amberlite IRC-50 significantly increased fecal potassium-42 excretion and considerably decreased the excretory ratio over that found on basal diet. Bentonite and Amberlite IRC-50 significantly decreased urinary potassium-42 excretion. In the *in vitro* studies with potassium-42, Table IV, bentonite had an affinity for potassium even in 0.1 N solutions of hydrochloric acid or sodium chloride. Vermiculite exhibited some preference for potassium

TABLE IV. Potassium-42 Remaining in Solution Expressed as % of Standard Solutions.

Treatment	Potassium-42 diluent			
	.1 N KCl	.1 N HCl	.1 N NaCl	H ₂ O
None	98	101	101	93
Vermiculite	98	97	86	37
Alfalfa	96	100	100	95
Beet pulp	95	101	95	75
Bentonite	91	85	80	58*
Amberlite IRC-50	99	100	99	17

Least significant difference between means at 1% level of confidence is 4.

* Supernatant contained suspended particles.

ions over sodium ions; however, appreciably more potassium-42 was removed when no other ions were present. Amberlite IRC-50 and beet pulp showed very little affinity for potassium-42 until no other ions were present.

In the rubidium-86 experiment, Table V, a significant reduction in urinary, fecal, and total rubidium-86 excretion and in excretory ratios was observed in rats fed potassium-deficient diets when compared to those fed comparable potassium-adequate diets. Rats fed vermiculite diets excreted significantly more rubidium-86 in the feces than did those fed the basal diets, even though no difference in total excretion of rubidium-86 was observed between diets containing equivalent amounts of potassium. Significantly more rubidium-86 was excreted by rats fed diets containing alfalfa, bentonite, and Amberlite

TABLE V. Influence of Some Organic and Inorganic Materials on Excretory Pattern of Rubidium-86 in Rats.

Treatment	Excretion*			Ratio of urinary /fecal Rb-86
	Urinary	Fecal	Total	
Basal + K	9.0	.9	9.9	10.0
" - K	1.5	.3	1.8	5.6
Vermiculite + K	5.8	4.0	9.8	1.5
" - K	.5	2.0	2.5	.3
Alfalfa + K	14.4	1.1	15.5	12.6
Beet pulp + K	9.4	2.0	11.4	4.8
Bentonite + K	5.4	9.5	14.9	.6
Amberlite IRC-50 + K	7.1	7.0	14.1	1.0
L.S.D.†	2.7	.6	1.8	

* Expressed as % of admin. dose/rat.

† Least significant difference between means at 5% level of confidence.

IRC-50 than those fed the basal diet. This increase with alfalfa occurred through urinary excretion, while with bentonite and Amberlite IRC-50 it occurred through fecal excretion. The order of fecal excretion of rubidium-86 was bentonite > Amberlite IRC-50 > vermiculite > beet pulp. As occurred with cesium-134, both bentonite and vermiculite significantly decreased urinary rubidium-86 excretion. The *in vitro* studies, Table VI, indicated that vermiculite and bentonite preferentially removed rubidium-86 from 0.1 N solutions of potassium chloride, hydrochloric acid or sodium chloride. Amberlite IRC-50

TABLE VI. Rubidium-86 Remaining in Solution
Expressed as % of Standard Solutions.

Treatment	Rubidium-86 diluent			H ₂ O
	.1 N KCl	.1 N HCl	.1 N NaCl	
None	98	100	100	91
Vermiculite	90	80	53	13
Alfalfa	98	99	99	97
Beet pulp	95	97	93	76
Bentonite	76	68	53	49*
Amberlite	96	99	97	14
IRC-50				

Least significant difference between means at 1% level of confidence is 3.

* Supernatant contained suspended particles.

and beet pulp did not appreciably remove rubidium-86 from solution until no other ions were present.

Comparisons of *in vitro* data with that found *in vivo* show a comparable order as found in distilled water, if one allows for a lower value for bentonite (closely approximating vermiculite) which would probably occur if no colloidal suspension were present.

Since all radionuclides were subcutaneously administered, the changes in excretory pattern of cesium-134, potassium-42 and rubidium-86 exhibited by the rat appear to be due to certain materials in the gut preferentially adsorbing nuclide during passage of the nuclide from body fluids into gut. If no materials are present in the gut to adsorb or remove these nuclides, most nuclides pass back into the body fluids and are either passed out through the kidneys or are recycled through the body and eventually reach the gut again and the cycle continued. This is substantiated

in part by the continued high fecal excretion occurring on some diets 48 and 72 hours after administration of nuclides to the rats.

Summary. These experiments demonstrated that absence of dietary potassium in basal or vermiculite diet decreased urinary and fecal excretion of cesium-134, potassium-42 or rubidium-86, as well as their excretory ratio within each diet, but the pattern between the 2 diets remained relatively the same. Vermiculite, alfalfa, beet pulp, bentonite, and Amberlite IRC-50 fed to rats had the property of influencing the excretory patterns of radionuclides used. The magnitude of influence these materials exerted upon urinary and fecal excretion of these radionuclides varied between nuclides. The results *in vitro* demonstrated that some of these materials exhibited an affinity for specific nuclides even though these materials were in minute quantities in 0.1 N solutions of potassium chloride, hydrochloric acid, or sodium chloride.

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Diffusion Times of Spermicidal Compounds. (22962)

CLARENCE J. GAMBLE. (Introduced by J. L. Gamble)
Department of Anatomy, Harvard Medical School, Boston, Mass.

Evaluation of contraceptive materials by observations of resulting pregnancy rates is difficult and time consuming. It is, moreover, uncertain because of unreported omissions or incorrectness of use. For this reason laboratory tests have been developed based on measurements of the power to immobilize sperm in human semen. To approach conditions in the postcoital vagina semen has been placed in contact with contraceptive preparations, and duration of motion of sperm observed (Baker *et al.*, 1, Davidson, 3, Gamble, 4). Large differences in actions of commercial contraceptive preparations have been observed.

To determine individual activities of chemical compounds used in such preparations, rates of immobilization by one of these tests (Gamble, 4) have been measured after incorporation in active jellies. The results of these assays are reported in this paper.

Materials used. Each compound was incorporated into a jelly having a consistency approximating that of commercial contraceptive jellies. Since it seemed possible that jellies of different types would release included compounds at different rates, 2 jelly-forming materials were used, each had, by itself, little or no immobilizing effect. Rice flour in a concentration of 20% water present, was used for all compounds except 15% lactic acid, which required 30% of flour for the desired consistency. Ten % tragacanth was used. *Compounds chosen.* Some compounds were among those in commercial contraceptive mixtures (Gamble, 4). Others were: acetic acid which has been advocated for contraceptive use with a tampon; zinc sulfocarbamate, and sodium tartrate, which have been incorporated in contraceptive foam tablets (Gamble, 8); hydroquinone (Baker, 2); and chlorhydrol and zephiran. *Concentrations tested.* Most compounds were tested in 2 concentrations. One termed "normal", was approximately the highest found in known

formulas of commercial contraceptive mixtures. The other, a "high" concentration, was about 10 or more times "normal", or a solution saturated at approximately 23°C. The concentrations used are given as % of water present. The more concentrated mixtures, tested to determine the possibility of increasing spermicidal activity, may well be too concentrated to be tolerated by human tissues, and are not suggested for contraceptive use. *Procedure.* Semen was secured from 6 medical students 22 or 23 years of age, at least 3 days after previous ejaculation. It was kept in an ice bath. Before it was 3 hours old a column of semen 5 mm long (4.8 to 5.2 mm) was placed in a glass tube of 2 mm inside diameter, in contact with a column of jelly 10 or more mm long. The tubes were sealed with paraffin and held in a horizontal position. Motion of sperm was observed through 10X objective and 10X eyepiece. Indirect illumination was by coiled filament of small headlight bulb placed at an angle of about 45° from the axis of microscope. Partial optical correction for the cylindrical surfaces was secured by smearing the specimen tube with immersion oil and placing it between a glass microscope slide and a cover glass. For convenience 2 parallel tubes were used to support the coverglass in horizontal position. Except during observation the tubes were kept in incubator at 37°C. Time was recorded from first contact of semen with jelly to immobilization of last sperm discoverable in the column between jelly and semen-air meniscus. Motion of sperms in semen around the hemispherical meniscus was disregarded. Spermicidal time with a given mixture varied with donor, ejaculate, age of ejaculate, and to a lesser extent with repetition of observation. Immobilization time of each compound was, therefore, measured in each of 2 jellies, with semen from 4 or more donors. The average of all tests with a given compound and jelly is recorded in the accompanying Table, except for

TABLE I. Spermicidal Times of Compounds in Rice or Tragacanth Jellies.

Compound*	Normal concentration			High concentration		
	%	Rice jelly, min.	Tragacanth jelly, min.	%	Rice jelly, min.	Tragacanth jelly, min.
NaCl				Sat.	31	32
"				30	29	24
"				20	42	40
"	10	110	70			
Sodium tartrate	10			Sat.	46	80
Quinine hydrochloride	5	120	100			
Phenylmercuric acetate				Sat.	100	110
" nitrate	.06	140	130	"	140	70
Trioxymethylene	.15	190	90	3.75	60	44
p-Diisobutylphenoxy-poly- ethoxyethanol	1.	190	220	12.5	130	170
Acetic acid	1.	230	160	10	80	60
p-Triisopropylphenoxy-poly- ethoxyethanol	1.25	260	190	12.5	80	110
Dextrose	45	300	310			
Chloramine T	2	320	180	12	90	120
Triethanolamine	10.5	330	210	50	100	100
Phenol	.24	360	200	5	90	80
Zinc sulfocarbolate				25	190	190
Chloroeresol	.15	420 + 1	280	Sat.	410	180
Hexylresorcinol				Sat.	420	160
Lactic acid	1.5	420 + 2	400	15	90	190
Methyl p-hydroxybenzoate	Sat.	420 + 2.6	320			
Sulfonated castor oil				Sat.	360	420 + 3.
Polyethylene glycol monoiso- octyl phenyl ether	.3	420 + 3.4	140	7.5	410	190
Dodecaethylene	5		420 + 1	†		70
Propyl p-hydroxybenzoate	Sat.	420 + 4	420 + 3			
Duponol C	.2	420 + 4.9	420 + 5	5	420 + 5	290
Zephiran chloride	.2	"	360	2.5	420 + .9	330
Hydroxyquinoline sulfate	.9	420 + 5	280	Sat.	220	210
Hydroquinone	1	"	320	10	80	80
Glyceryl monoricinoleate	1	"	420 + 2.6	10	420 + .2	420 + 2
Hydroxyquinoline benzoate	.9	"	420 + 3	Sat.	290	260
Aluminum acetotartrate	3.125	"	420 + 5	30	180	180
Aminoacetic acid	.1	"	"	10	420 + 5	420 + 5
p-tert amylphenol	.2	"	"	Sat.	"	420 + 3.2
Benzethonium chloride	.2	"	"	5	230	280
Butyl p-hydroxybenzoate	Sat.	"	"			
Cetyl alcohol	1.	"	"	Sat.	420 + 0.5	420 + 5
Cetyl dimethyl benzyl am- monium chloride	.25	"	"	"	420 + 5	420 + 3
Chlorhydrol	10	"	"	100	360	230
Chlorothymol	Sat.	"	"			
Dioctyl sodium sulfosue- cinate	.5	"	"	Sat.	"	420 + 1.5
Nonaethylene glycol mono- laurate	1.	"	"			
p-Chloro-m-xlenol	Sat.	"	"			
Ricinoleic acid	"	"	"			

* Ranked according to times of normal concentrations in rice jelly.

† Pure compound without water or tragacanth.

The figures following + give mm of semen column in which sperm continued in motion. Stand. dev. of each time given is approximately 17%.

preparations that showed motility in one or more tests at the end of 7 hours. For these the median value is used. If this exceeded 7 hours, length in mm of semen column still showing motility is also given.

Results. Immobilization times in the Table

of more than 50 minutes have been rounded off to nearest 10 minutes. Results are approximately the same with each jelly-forming material. Of comparable averages or medians 35 are shorter with tragacanth, 15 with rice jelly, and 18 are equal. This suggests that

tragacanth may release spermicidal compounds a little more rapidly, or that it may have slight additive spermicidal effect.

Correlation of ranking by 2 materials is significant, being 0.60 for "normal" concentrations, and 0.61 for those that were "high".

Spermicidal times of pure compounds were relatively long, when compared to those of commercial contraceptive mixtures, of which the median time of 87 brands was 226 minutes (Gamble,5). Only 5 of the 35 compounds in contraceptive jellies, creams, or suppositories, had times less than this in "normal" concentration in rice jelly. The greater activity of commercial mixtures may be due in part to the fact that they contain more than one compound having spermicidal activity.

Even when "high" concentrations are considered, however, some of which are many times "normal" strength, the spermicidal time of pure compounds is not much shorter than commercial mixtures. Only 18 of 33 tested in rice jelly were below 226 minutes, the median of commercial mixtures.

The shortest spermicidal time was found with NaCl. In rice jelly containing 10% salt, sperm were immobilized in 110 minutes, and when jelly was saturated, in 31 minutes.

Commercial contraceptive materials containing NaCl had previously been found more active than most (Gamble,6).

Saturated sodium tartrate had relatively low spermicidal time. This is the end product after exposure to moisture of contraceptive which were clinically effective (Gamble,8).

Quinine hydrochloride, 5%, was also active, 120 and 100 minutes in rice and tragacanth. This activity was greater than a suppository, containing 7.74% of quinine, 401 minutes (Gamble,7), perhaps because of greater solubility of the salt.

Paraformaldehyde, which was present in 9 of 87 commercial mixtures previously studied, and phenyl mercuric compounds which occurred in 20 of 87 preparations (Gamble,4) were relatively active.

Dextrose in 45% concentration had median time of 300 minutes in rice jelly and 310 in tragacanth. This was much longer than the corresponding 110 and 70 minutes for 10%

NaCl which has about the same osmotic effect, suggesting that the spermicidal action of NaCl is not entirely osmotic.

Less extensive tests with semen from 2 donors showed that saturated KCl, with time of 50 minutes, and saturated sodium sulfate with time of 150 minutes were less active than NaCl or sodium tartrate.

Discussion. In clinical contraception semen is presumably deposited in droplets which are partly or completely surrounded by contraceptive mixture. The time required for immobilization of sperm in the droplets is dependent upon a number of unrelated factors: 1. A higher concentration of immobilizing compound in the cream, jelly or suppository will result in shorter spermicidal time. Concentration is limited by solubility of the compound, and by possibility of damage to tissues exposed to the mixture. 2. A compound which diffuses rapidly from surface of mixture into the contiguous semen will stop motion of sperm more rapidly. Such diffusion is faster for compounds of low molecular weight, and may depend upon the nature of the surface of jelly-forming material. 3. The greater the area of semen exposed to contraceptive mixture, the more rapid is diffusion of the compound into the semen. This is dependent upon unknown size of semen droplets, which is probably independent of the nature of contraceptive mixture. 4. The shorter the distance from surface of semen droplets to center, the more rapidly will the last sperm be immobilized. As with surface area this distance is dependent upon unknown and unreproducible size of semen droplets. 5. The more rapidly an immobilizing compound is transported throughout the droplet, the shorter will be immobilization time. Molecular diffusion will contribute to this, but in droplets of the size to be expected in post-coital semen, diffusion is much less effective than convection currents. This was shown by the finding that when semen was placed above contraceptive jelly in a vertical tube, the zone in which sperm had been immobilized increased with extreme slowness in comparison with the same preparation in a horizontal tube (Gamble,4).

When these factors in clinical action of

contraceptive compounds are considered, it is clear that ranking of presumed clinical activity based upon spermicidal times, is not necessarily that which would be found, could immobilization be observed in a postcoital droplet of semen. The droplets may well be smaller than the column 5 mm long and 2 mm in diameter used in the test, and are exposed to contraceptive material over their entire surface. Rapidly diffusing compounds may, therefore, be more effective clinically than ranking by this test indicates.

As distance to center of droplet is probably less than 5 mm distance from jelly to the most distant sperm, ability to cause convection may be less important in clinical use.

In spite of these unavoidable divergences, our laboratory test appears to provide a useful approximation to postcoital conditions. It is reasonable to conclude that the compounds with a relatively short spermicidal time in concentration which does not cause irritation, are desirable for inclusion in contraceptive mixtures.

Sodium chloride with other compounds. From the above, it was inferred that NaCl in high concentration would produce active convection in the column of semen, and such convection was observed (Gamble, 4). It, therefore, seemed possible that spermicidal activity of a compound might be greatly increased if sodium chloride were added to aid in spreading it through the semen column. Tragacanth jellies were, accordingly, prepared containing a "normal" concentration of each of the 15 compounds found most active, together with 10% NaCl, and tested with semen from 2 donors. The average spermicidal time was, in most instances, slightly less than that of 10% NaCl alone. For no substance, however, was the time found with added 10% NaCl as short as that of 20% NaCl. Consequently, the hope of finding an unusually short spermicidal time resulting from including sodium chloride in a jelly with other substances was not fulfilled.

Summary and Conclusions. 1. To estimate comparative contraceptive effectiveness of chemical compounds, under conditions approximating those of clinical contraception, each was incorporated in rice flour or tragacanth jelly. Time to immobilize all sperm in a column of human semen in contact with jelly was then observed. 2. The time of various compounds in commercial contraceptive mixtures, and of others, was compared in this way, both in concentrations used in commercial preparations and in concentrations 10 or more times as great. 3. NaCl had the shortest time observed, probably due in part to convection currents it creates in semen. Time of saturated sodium tartrate was also short. 4. Only a small proportion of compounds in concentrations used in contraceptive mixtures showed times shorter than the median of a series of commercial preparations. 5. Addition of compounds used in contraceptive mixtures to 10% NaCl jellies did not result in times much shorter than that of NaCl alone. From these results it is believed that in contraceptive mixtures greater spermicidal activity is to be expected from compounds of low molecular weight. Diffusion of such compounds from jelly into semen, which must necessarily precede spermicidal activity, will be more rapid than that of heavier compounds. Sodium chloride and sodium tartrate, which are nontoxic, appear desirable compounds for contraceptive mixtures.

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Glycogen Stores in Glucagon-Treated Rats. III. Effect of Adrenalectomy and Hypophysectomy.* (22963)

GIORGIO GALANSINO, GUIDO POZZA,[†] ERMINIO COSTA[‡] AND PIERO P. FOÀ

Department of Physiology and Pharmacology, The Chicago Medical School, Chicago 12, Ill.

Intraperitoneal injections of glucagon and of epinephrine cause an immediate and marked reduction in liver glycogen(1,2), due to an increase in phosphorylase activity(3,4). This glycogenolytic phase is followed, 24 hours after glucagon and 2 hours after epinephrine, by marked increase in liver glycogen(1,5,6) and is accompanied by increase in adrenal weight and decrease in adrenal ascorbic acid. In alloxan diabetic rats, the glycogenolytic effect of glucagon and epinephrine appears to be unimpaired, but no secondary increase in liver glycogen can be obtained(7). These results suggest that the rebound phenomenon requires the presence of 1) an intact insulin-secreting system capable of being stimulated by glucagon or epinephrine hyperglycemia and 2) the adrenal glands, possibly participating in a permissive manner. This paper describes the effects of glucagon and epinephrine on liver and muscle glycogen in adrenalectomized and in hypophysectomized rats with and without cortisone or insulin treatment.

Materials and methods. Two hundred sixteen male Sprague-Dawley albino rats weighing 150-200 g were used. Of these, 139 were adrenalectomized,[§] 77 were hypophysectomized.^{||} All animals were kept on diet of Purina checkers *ad libitum*. During the first postoperative week the diet of hypophysectomized rats was ground and moistened with water to help overcome anorexia. Adrenalectomized animals drank 1% glucose in saline instead of water, except when treated with

cortisone. All animals were fasted 24 hours before the experiment. Glucagon[¶] (Eli Lilly and Co., lot No. 208-158B-197: 2.5 or 5 mg/kg) or epinephrine (0.15 mg/kg) and glucose (1 or 2 g/kg in 5% solution) were injected intraperitoneally at the beginning of the experiment. Cortisone (1 or 5 mg/kg) was injected intramuscularly daily for 10 days up to 48 hours before the experiment. Insulin (Ultralente Lilly, 0.05 u/rat) was injected subcutaneously in a single dose at the beginning of the experiment and was followed by a second injection of glucose 5 hours later. After 20 minutes, 2 or 24 hours, the animals were decapitated, samples of liver and of left gastrocnemius muscle were removed as quickly as possible and placed in tared centrifuge tubes containing 30% KOH. Glycogen was isolated and hydrolyzed by the method of Good, Kramer and Somogyi(8), and resulting glucose determined according to Nelson(9). Each experiment consisted of an experimental group of rats treated with glucagon or epinephrine and a control group of rats treated with saline. The statistical significance of the difference between mean experimental and control values was calculated according to Fisher(10).

Results. Results are presented in Table I. In adrenalectomized rats the results were as follows: 1) Fasting liver glycogen was very low and no significant changes were noted 2 hours after injection of glucagon. Twenty-four hours after the injection there was a significant but small rise in liver glycogen. 2) Treatment with cortisone caused a partial restoration of liver glycogen toward normal values. This restoration was more pronounced

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[†] Research Fellow—Present address: Istituto di Clinica Medica, Università di Milano, Italy.

[‡] Research Fellow—Present address: State Research Hospital, Galesburg, Illinois.

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^{||} Hypophysectomy was performed through the courtesy of Dr. I. Bunding of the Armour Laboratory.

[¶] Glucagon and Insulin were gifts of Drs. O. K. Behrens and M. A. Root of the Lilly Research Laboratories.

** Three control animals gave abnormally low muscle glycogen values. If these are eliminated, the reduction due to epinephrine becomes statistically significant, as indicated by values in parentheses.

TABLE I. Effect of Glucagon and Epinephrine on Liver and Muscle Glycogen of Adrenalectomized (Adx) and Hypophysectomized (Hyphex) Rats Fasted 24 Hr (mg/100 g of Fresh Tissue).

No. and type of rats	Time inj., hr	Glucagon, mg/kg	Corti- son, mg/kg	Epine- phrine, mg/kg	Insulin, U/rat	Glucose, g/kg	Glycogen			
							Liver		Muscle	
							Avg $\pm$ S.E.	% change	Avg $\pm$ S.E.	% change
14 Adx*						2	30		277	
13 †	2	2.5				"	31	+	363	+31§
10						"	17		356	
10	24	2.5				"	27	+	401	+12§
8(5)†						"	38		277(358)	
8	1½			.15		"	28	-	185	-30§(-48)‡
8	2			"		"	22		356	
8	2			"		"	21	-	357	+7§
6						"	184			
8	24	5	1			"	220	+	25§	
7	"	5	5			"	630	+	40‡	
7	"	5	"			"	878			
6			"			"	261		210	
6	1½		"	.15		"	19	-	196	-7§
6	"		"			"	252		276	
6	2		"	"		"	796	+	126	-54‡
8 Hyphex						"	40			
5	"	5	"			"	39	-	2.5§	
7						"	22			
4	24	5	5			"	25	+	13§	
6	2	5	"			"	642	-	411	-3§
6	"	5	"			"	32	-	397	
12†	24	5	"			"	571	+	411	+18§
12†	"	5	"			"	772	+	486	
6			"			"	642	-	411	-55‡
5	1½		"	.15		"	74	-	185	
6	"		"	"		"	642	-	411	-78‡
6	2		"	"		"	196	+	271	
6	24	5	"	.05		4	21	+	290	-10§
6	"		"	"		"	49	+	21	

* Muscle values for 8 rats.

† Muscle values for 7 rats.

‡ Muscle values for 6 rats.

§ P > 0.05.

|| P $\leq$ 0.05.

¶ Three control animals gave abnormally low muscle glycogen values. If these are eliminated, the reduction due to epinephrine becomes statistically significant, as indicated by values in parentheses.

in rats receiving cortisone in daily doses of 5 mg/kg than in rats receiving 1 mg/kg. 3) Twenty-four hours after injection of glucagon in adrenalectomized cortisone-treated rats there was a significant increase in liver glycogen above control values. This increase was statistically significant only in rats treated with cortisone in daily doses of 5 mg/kg. 4) Glucagon caused no significant changes in muscle glycogen. 5) Epinephrine caused a temporary decrease in liver and muscle glycogen. This decrease was not statistically significant. 6) In adrenalectomized cortisone-treated animals, epinephrine caused a marked and significant decrease in liver glycogen, followed 2 hours later by a significant decrease in muscle glycogen and by a marked and significant increase in liver glycogen above the initial values.

In hypophysectomized rats, the results were as follows: 1) In animals receiving no cortisone, liver glycogen was very low and did not change significantly 2 or 24 hours after injection of glucagon. 2) In rats treated with cortisone, liver glycogen decreased significantly 2 hours after injection of glucagon and returned to, but not above, initial values 24 hours later. 3) Twenty-four hours after injection of glucagon, liver glycogen of insulin-treated hypophysectomized rats was significantly increased. 4) Muscle glycogen was not changed significantly by glucagon injections. 5) Twenty minutes after injection of epinephrine there was a marked reduction in liver and muscle glycogen. Both were still significantly below initial values 2 hours thereafter.

Discussion. Liver glycogen of fasted, untreated adrenalectomized rats was many times lower than that of fasted normal animals, as reported by other investigators(5). Injection of glucagon or epinephrine did not cause any immediate further decrease, perhaps because an irreducible minimum had already been reached. The delayed increase in liver glycogen averaged 10 mg/100 g of tissue and, although statistically significant, was inadequate to raise the values above those generally found in adrenalectomized rats. Cortisone treatment increased fasting liver glycogen and restored the glycogenolytic effect. In

these animals glycogen rebound averaged 248 mg/100 g of tissue. These results suggest that adrenals are a factor in the rebound due to glucagon as they are in that due to epinephrine(11). Recently, Root(12) reported that daily glucagon injections produced an increase in liver glycogen of rats adrenalectomized 48 hours before beginning of treatment. These animals may be comparable to the adrenalectomized animals treated with cortisone up to 48 hours before the experiment and may have sufficient residual adrenal cortical hormones to permit glycogen rebound. Hypophysectomy, like adrenalectomy, reduced liver glycogen and the immediate glycogenolytic effect of glucagon was detectable only in those animals in which the glycogen stores had been replenished by cortisone, although it still remained far below normal values. No secondary liver glycogen increase was noted in hypophysectomized rats whether treated with cortisone or not, while a marked and significant rebound was noted in hypophysectomized rats treated with insulin. These results suggest that the delayed glycostatic effect of glucagon is greatly diminished in the absence of the adrenals or when the adrenal cortical activity is reduced to a minimum by hypophysectomy. It is possible that a pituitary factor, perhaps growth hormone, capable of maintaining a normal insulin function(13) may be necessary for this phenomenon, in agreement with the observation that growth hormone has no glycostatic effect in alloxan diabetic rats(14). The results described in this and in preceding papers of this series show that the immediate liver glycogenolysis induced by glucagon is followed by marked increase in liver glycogen in normal rats, in adrenalectomized rats treated with cortisone and in hypophysectomized rats treated with insulin, but not in alloxan-diabetic rats and in untreated adrenalectomized or hypophysectomized animals. It is believed, therefore, that this rebound is not a direct effect of glucagon, but the result of a secondary secretion of insulin, a hormone known to increase liver glycogen(15,16). Glucagon hyperglycemia causes an almost immediate secretion of insulin(17), while the effect of insulin on liver glycogen is slow(18,

19). In this manner a quick acting hormone like glucagon could cause delayed effects. The glycogenolytic effect of epinephrine in muscle of adrenalectomized rats was not modified significantly by cortisone treatment, in agreement with the belief that epinephrine acts directly on muscle phosphorylase. On the other hand, glucagon, which does not stimulate muscle phosphorylase, does not seem to exert any direct action on muscle glycogen. The occasional increase in muscle glycogen observed in normal animals following glucagon injection(7) may be the result of adrenal cortical stimulation, as shown by the fact that it does not occur in adrenalectomized and in hypophysectomized rats. The only changes in muscle glycogen in these animals were those due to the well known glycogenolytic effect of epinephrine(1,5,6).

Summary and conclusions. The effect of glucagon and of epinephrine on liver and muscle glycogen of adrenalectomized and hypophysectomized rats with or without treatment with cortisone or insulin was studied.

1) Epinephrine caused an immediate decrease in muscle glycogen in all cases and a decrease in liver glycogen in animals treated with cortisone. 2) In untreated adrenalectomized rats, liver glycogen was very low and further decrease due to epinephrine was not significant. No secondary increase in liver glycogen was noted. 3) Two hours after injection of glucagon, no significant changes in liver and muscle glycogen of untreated adrenalectomized or hypophysectomized animals were noted. 4) Twenty-four hours after the injection of glucagon in untreated adrenalectomized rats there was a significant, but very small increase in liver glycogen. Muscle glycogen was unchanged. No significant changes were noted in liver and muscle glycogen of untreated hypophysectomized rats. 5) In cortisone treated adrenalectomized or hypophysectomized rats, glucagon caused a significant decrease in liver glycogen, 2 hours after injection. 6) No secondary increase in liver glycogen was noted in cortisone treated hypophysectomized rats. 7) Glucagon caused a secondary increase in liver glycogen in cortisone treated adrenalectomized rats and in hypophysectomized rats treated with insulin. 8) No significant changes in muscle glycogen of treated or untreated rats were noted following glucagon injection. 9) The results are consistent with the hypothesis that glycogenolysis and hyperglycemia are the primary effects of glucagon and epinephrine and that the delayed increase in liver glycogen and occasionally of muscle glycogen, observed after injection of these hormones, is the result of a secondary secretion of insulin aided by the "permissive" action of the adrenal cortex. 10) The importance of time factors in this "rebound" phenomenon is emphasized.

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